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Title: Interactions between extracellular Hsp72 and blood cells

Date: December 2010

Originally published as: University of Liverpool PhD thesis

Example citation: Williams, H. (2010). *Interactions between extracellular Hsp72 and blood cells*. (Unpublished doctoral dissertation). University of Liverpool, United Kingdom.

Version of item: Submitted version

Available at: <http://hdl.handle.net/10034/277691>

# **Interactions between extracellular Hsp72 and blood cells**

Thesis submitted in accordance with the requirements of  
The University of Liverpool  
for the degree of Doctor of Philosophy by

**Helen Williams**

**December 2010**

**University of Chester**



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## **Declaration**

The work presented in this thesis is original and has not been submitted previously in support of any qualification or course.

**Signed:**

**Date:**



## **Acknowledgements**

This research was carried out in the Department of Biological Sciences at the University of Chester and was supported by a Gladstone Bursary. My sincere gratitude is due to the University of Chester for their funding of this study and all of my colleagues who made it possible for me to complete this thesis.

I would like to acknowledge the following people who have advised and supported me throughout this time:

My supervisors: Professor John Williams and Dr Elyse Ireland for all their advice, support and excellent guidance throughout my Ph.D.

Fellow Researchers: Dr Robert Coleman, Dr Francesca Leoni, Dr Nina Dempsey and Dr Ola Altaie. I would like to thank you all for your help and advice, support and good friendship throughout the years.

Finally, I would like to express my gratitude to my family, especially my Mum and Dad, and Martin for all their constant support, generosity and sincere encouragement during this time.





## Abstract

In recent years, compelling evidence has accumulated suggesting heat shock proteins (HSPs) which are generally believed to be localised and functioning mainly within eukaryotic cells as cyto-protective molecular chaperones, are also localised in the extracellular milieu. Depending on their localisation, on the cell surface (membrane-bound or embedded), or in the peripheral circulation, extracellular HSPs may induce apoptotic cell death, or in contrast protect cells from cell damage and/or cell death when exposed to cellular stress, or may even elicit a stimulatory effect on the innate immune response including cell activation and cytokine secretion. Hence, the localisation of intracellular and extracellular HSPs appears to be critical in determining their roles in terms of stimulating cell death, cyto-protection, or immune activation under normal physiological conditions and following exposure to stress stimuli.

This thesis describes the intracellular expression, up-regulation, and cell surface localisation of endogenous HSPs: Hsp27, Hsp60, Hsp72 and Hsp90 by flow cytometry, fluorescence microscopy and Western blotting, under control conditions and in response to environmental stress using *in vitro* and *ex vivo* models with the intention of determining their physiological roles. The ability of extracellularly administered HSPs (Hsp70 and Hsp72) to protect cultured U937 cells *in vitro* or peripheral primary human leukocytes or erythrocytes *ex vivo* from various stress stimuli was demonstrated and was found to be dependent on surface binding and/or internalisation *via* scavenger receptors (SRs) or phosphatidylserine (PS), which could be blocked by receptor specific ligands. Extracellular HSPs were also shown to be able to stimulate an immune response through the induction of U937 monocyte differentiation into macrophages as evidenced through the up-regulation of the surface receptors: CD36, SR-A1 and CD91 analysed by flow cytometry. These proteins were able to stimulate TNF- $\alpha$  and IL-10 production and secretion by U937 macrophages, shown by ELISA, and chemotactic properties were demonstrated using Boyden chambers.

The cyto-protective and immune regulatory effects of extracellular HSPs have potential therapeutic value as treatments in a wide variety of clinical situations.

## **Publications**

Williams, H., Ireland, H. E. & Williams, J. H. H. (2010). Interactions between SR-A1 and Hsp72 (HspA1A) increases erythrocyte longevity during physiological stress by stabilising the membrane. (In Preparation).

Williams, H., Ireland, H. E. & Williams, J. H. H. Hsp72 activates macrophage differentiation and is a chemoattractant for leukocytes. *Inflammation Research*, 59 (supplement 1): A 211.

(Oral poster presentation at 8<sup>th</sup> World Congress on Trauma, Shock, Inflammation and Sepsis – TSIS2010, Munich, Germany. March 2010)

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## Abbreviations

AD	Alzheimer's disease
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease-activating factor 1
APC	Antigen presenting cell
ATP	Adenosine-5'-triphosphate
AU	Absorbance units
BSA	Bovine serum albumin
CGA	Chromogranin A
DC	Dendritic cell
DIABLO	Second mitochondria derived activator of caspase
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DRMs	Detergent-resistant microdomains
dH <sub>2</sub> O	Distilled water
ELISA	Enzyme linked immuno-sorbent assay
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FBS	Foetal bovine serum
FCS	Forward scatter
FITC	Fluorescein isothiocyanate
Grp	Glucose regulating protein
HD	Huntington's disease
HI-FBS	Heat inactivated serum
HI-RPMI	RPMI-1640 medium with 10 % heat inactivated serum
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Hsc	Heat shock cognate
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein

Hsp70	Bovine heat shock protein 70
Hsp72	Human heat shock protein 72
IAP	Inhibitor of apoptosis protein
IL	Interleukin
INF- $\gamma$	Interferon-gamma
IRF	Interferon response factor
kDa	Kilo Dalton
LDL	Low-density lipoproteins
LPS	Lipopolysaccharide
malBSA	Maleylated bovine serum albumin
M-CSF	Macrophage colony-stimulating factor
MFI	Mean fluorescence units
MHC	Major histocompatibility complex molecules
mRNA	Messenger ribonucleic acid
MVBs	Multivesicular bodies
MWCO	Molecular weight cut-off
NaCl	Sodium chloride
NF- $\kappa\beta$	Nuclear factor kappa-beta
NK	Natural killer
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD	Parkinson's disease
PE	Phycoerythrin
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
PI	Propidium iodide
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
Poly (I)	Polyinosinic acid
PRR	Pathogen recognition receptor
PS	Phosphatidylserine



10 % RPMI	RPMI-1640 medium with 10 % foetal bovine serum
RA	Rheumatoid arthritis
RAPs	Receptor associated proteins
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SLE	Systemic lupus erythematosus
SRs	Scavenger receptors
SSC	Side scatter
TAP	Transporter-associated antigen processing
TH-1	T-helper cells type 1
TH-2	T-helper cells type 2
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRADD	TNF receptor-associated death domain
VIP	Vasoactive intestinal peptide
v/v	Volume by volume
w/v	Weight by volume
$\Lambda$	Wavelength
$\lambda$ EM	Emission wavelength
$\lambda$ EX	Excitation wavelength

## **Chapter 1**

### **Introduction**

#### **1.1 Rationale for study**

Until recently heat shock proteins (HSPs) were not considered as molecules of immunological importance, rather intracellular proteins with critical roles in maintaining cellular homeostasis and in protecting the cell against potentially harmful or detrimental stimuli. However, the discovery of their existence in the extracellular milieu (Tytell, Greenberg & Lasek, 1986; Hightower & Guidon, 1989), both basally and in response to cellular stress, and as membrane-bound or embedded proteins on tumour (Multhoff et al. 1995) and virally-infected (Chouchane et al. 1994) cells has led to the increasing interest in the role(s) of extracellular HSPs. In particular, the stress-inducible heat shock protein 72 (Hsp72), has been described in several stressful situations in the extracellular environment, including physical exercise (Febrenbach et al. 2000; Walsh et al. 2001; Febbraio et al. 2002), fever (Oehler et al. 2001), infection (Njemini et al. 2003; Moehler et al. 2003; Njemini et al. 2007), or psychological stress (Campisi & Fleshner, 2003a; Campisi, Leem & Fleshner, 2003b), where it has been proposed as an ancestral ‘danger signal’ that alerts the immune system to danger and promotes the generation of immunity and the stress resistance of immune cells (Matzinger, 2002; Campisi & Fleshner, 2003a; Campisi et al. 2003b).

The immune system elicits a potent response to endogenous danger signals, produced or released by cells undergoing stress, damage or abnormal cell death, through the activation of innate and adaptive immune responses (Feder & Hofmann, 1999; Matzinger, 2002). Recent studies reveal extracellular Hsp72 to stimulate such responses through binding to a variety of cellular receptors particularly on antigen-presenting cells (APCs) (Thériault et al. 2005; Thériault et al. 2006), inducing the production and secretion of inflammatory cytokines (Basu et al. 2000b; Asea et al. 2000b; Campisi et al. 2003b; Svensson et al. 2006), chemokines (Lehner et al. 2000), and co-stimulatory surface molecules (Banchereau et al. 2000), inducing dendritic cell (DC) maturation (Gallucci, Lolkema & Matzinger, 1999; Basu et al. 2000b; Somersan et al. 2001) and thus signalling tissue damage, initiating inflammation and immunity.

It has also been well established that the immune system can generate antibodies to both human and bacterial HSPs, which have been implicated in several autoimmune diseases including rheumatoid arthritis (RA) and diabetes (Mapp, Grootveld & Blake, 1995; Macht et al. 2000; Finotti & Pagetta, 2004; Hunter-Lavin et al. 2004a) and various neurodegenerative diseases including Alzheimer's disease (AD) and Huntington's disease (HD) (Sakahira et al. 2002; Tiraboschi et al. 2004; Merienne et al. 2003). HSPs have been found to play a fundamental role in the pathology of health and a number of human diseases and disorders (Wick, Knoflach & Ku, 2004; van Eden, van der Zee & Prakken, 2005). This is primarily due to the inter-species high sequence homology of 50 % between microbial HSPs and endogenous mammalian HSPs derived from abnormal, damaged or stressed cells. This has led to the suggestion that there is a link between the immune response to infection and the development of autoimmunity (Lamb et al. 1989). The observations that HSPs when found in the extracellular environment can directly or indirectly elicit potent immunoregulatory functions requires a new thinking to be established on the roles of HSPs and anti-HSPs in autoimmunity, neurodegenerative disease and other conditions.

The work that will be presented in this thesis explores the potential function(s) of a number HSPs, in particular Hsp72, when located inside the cell, on the cell surface (membrane-bound or embedded), or in the extracellular milieu. The role of HSPs, in terms of cyto-protection, a potent inducer of apoptotic cell death, and/or immune regulation, under normal physiological conditions and in response to various stress stimuli using both cultured and human peripheral blood cells will be investigated.

## **1.2 Heat shock proteins**

The existence of HSPs were first reported by Ritossa and colleagues over forty years ago (Ritossa, 1962), when they noted odd puffing patterns and an unusual profile of gene expression in the polytene chromosomes of *Drosophila melanogaster* larval salivary gland cells exposed to elevated temperature (30°C) and the metabolic inhibitor dinitrophenol (Ritossa, 1962). More than a decade later it was revealed that these genes led to the increase in the transcription and

translation of proteins with molecular weights of 27 and 70 kDa (Tissieres, Mitchell & Tracy, 1974), and these proteins were subsequently named Hsp27 and Hsp70 (Tissieres et al. 1974). During the mid 1980s the study of HSPs dramatically increased and investigators found HSPs expressed in the majority of all living cells, including prokaryotic and eukaryotic species, inhabiting nearly all subcellular compartments (Lindquist & Craig, 1988; Hartl, 1996). These proteins were also found to be highly evolutionarily conserved (Boorstein, Ziegelhoffer & Craig, 1994), suggesting the structures of the proteins in humans are extremely similar to those of the equivalent molecules found in simpler forms of life (Eales, 1997), with the exception of the small HSPs, which display only limited homology (Ciocca et al. 1993a).

More recently, the term stress protein has been used in the recognition that in addition to heat shock, other environmental (UV radiation, heavy metals and amino acids), biological (bacterial, parasitic infections, fever, glucose starvation) and physiochemical agents (toxic substances) stimulate the synthesis of intracellular HSPs in most plant and animal systems (Parsell & Lindquist, 1993; Morimoto & Santoro, 1998; Ménoret et al. 2002). Stress induces damage at the molecular, cellular and organismal level (Soti & Csermely, 2007), and the up-regulation of HSPs enable the cell and organism to survive and recover from such conditions by maintaining normal cellular function under non-ideal conditions in a process known as the stress response. HSPs fulfil this vital function through their ability to interact with exposed hydrophobic stretches of the amino acid side chains of partially unfolded, misfolded or denatured polypeptides and proteins, produced as a result of protein folding or assembly events which may have been compromised under conditions of stress (Parsell & Lindquist, 1993; Tavaría et al. 1996; Bukau & Horwich, 1998; Morimoto & Santoro, 1998), to prevent aggregation of aberrantly folded proteins, further denaturation and/or cell death. This interaction results in the stabilisation, correct folding or refolding of the denatured polypeptides and proteins *via* hydrolysis of adenosine-5'-triphosphate (ATP) (Fink, 1999), or are degraded by ubiquitination or lysosomal proteolysis if the unfolding was irreversible (Fink, 1999). Furthermore the up-regulation of HSPs also facilitates the synthesis of new proteins, to replace those that were damaged under conditions of cellular stress. However, the induction of HSPs has to be tightly controlled, because their

persistent high presence would adversely affect protein homeostasis and intracellular functions, resulting in inappropriate growth control and possibly the development of cancerous cells (Pockley, 2003a). In mammalian cells, the HSPs induced by stress and primarily involved in the stress response are Hsp110, Hsp90, Hsp72, Hsp60, Hsp40 and members of the small HSPs, typified by Hsp27 (Table 1.1).

Despite their designation as stress proteins, it became clear shortly thereafter the discovery of heat-inducible proteins that in most cell types under normal physiological conditions, all HSP families also encode constitutively expressed members, named the heat shock 'cognate' proteins (Tavaria et al. 1996), which are expressed at high concentrations reaching 1-5 % of total cellular protein (Soti & Csermely, 2007). Constitutive HSPs are differentially expressed and/or regulated during the cell cycle and during development and differentiation (Ciocca et al. 1993a). They are of functional importance in the biology and physiology of the unstressed cell, particularly in protein homeostasis by regulating protein folding quality control (Soti & Csermely, 2007). In the cytosol, constitutive HSPs function as molecular chaperones and facilitate the synthesis and the early stages of folding and assembly of a wide variety of nascent polypeptides and proteins, to prevent inappropriate protein aggregation and to mediate the translocation and transportation of newly synthesised proteins from the cytoplasm into different intracellular compartments (Hartl, 1996; Bukau & Horwich, 1998). In addition, HSPs have been shown to participate in the regulation of transcription factors and protein kinases and in the stabilisation of receptors and signal transduction molecules (De Maio, 1999).

### **1.2.1 Heat shock protein families**

Mammalian HSPs are traditionally classified into five subfamilies according to their approximate molecular size in kDa and gene sequence homology. The families include: HSP100, HSP90, HSP70, HSP60 and the small HSPs, and each family contain both constitutive and stress-inducible members, for example the HSP70 family contains members Hsc70 (constitutively expressed) and Hsp72 (stress-inducible). They are expressed not only in the cytosol but within several cellular organelles including the mitochondria, endoplasmic reticulum (ER) and nucleus, and each family perform different intracellular functions as

demonstrated in Table 1.1 (Srivastava, 2002a). However, it is apparent when examining the HSP families that their nomenclature can be somewhat confusing at times and with their ever expanding members, due to sequencing of the human genome, has led to inconsistencies in their nomenclature whereby HSPs in publications are referred to by several different names, suggesting comparative analysis to be problematic. Recently, Kampinga et al. (2009) proposed a new novel nomenclature for human HSP families based upon the more consistent nomenclature assigned by the HUGO Gene Nomenclature Committee and used in the National Centre of Biotechnology Information Entrez Gene database for heat shock genes (Kampinga et al. 2009). This new system is based upon the fact that the gene name can be used for the protein produced, as demonstrated in Table 1.2.

This study focuses mainly on a member of the HSP70 superfamily: specifically Hsp72, in other words HSPA1A, however for the purpose of this thesis these proteins will be named throughout based on the old nomenclature system: Hsp72.

**Table 1.1: Classification of HSPs, their localisation and function.**

<b>HSP Name</b>	<b>Molecular Weight (kDa)</b>	<b>Localisation</b>	<b>Function</b>
Small HSPs	15-40	Cytosol, Nucleus	Cytoskeletal stabilisation
HSP60	58-65	Cytosol, Mitochondria	Protein folding, Prevention of aggregation
HSP70	66-78	Cytosol, Mitochondria, ER	Protein folding, Protection against stress, Down-regulation of HSF1 activity
HSP90	82-90	Cytosol, ER	Prevention of aggregation, Maintenance of HSF1
HSP100	97-120	Cytosol, Nucleus, Mitochondria	Thermotolerance, Protein refolding

Adapted from (Pockley, 2003a) and (Srivastava, 2002a)

**Table 1.2: The new classification of HSPs and related chaperone genes in the human database (HSPs referred to in this thesis).**

	Gene Name	Protein Name	Old Names
<b>HSPB</b>			
<b>1</b>	<i>HSPB1</i>	HSPB1	Hsp25, Hsp27, Hsp28
<b>DNAJ</b>			
<b>1</b>	<i>DNAJB1</i>	DNAJB1	Hsp40
<b>HSPD</b>			
<b>1</b>	<i>HSPD1</i>	HSPD1	Hsp60
<b>HSPA</b>			
<b>1</b>	<i>HSPA1A</i>	HSPA1A	HSP70-1; Hsp72; HSPA1
<b>2</b>	<i>HSPA1B</i>	HSPA1B	HSP70-2
<b>3</b>	<i>HSPA1L</i>	HSPA1L	Hum70t; hum70t; Hsp-hom
<b>4</b>	<i>HSPA2</i>	HSPA2	Heat-shock 70kDa protein-2
<b>5</b>	<i>HSPA5</i>	HSPA5	BIP; GRP78; MIF2
<b>6</b>	<i>HSPA6</i>	HSPA6	Heat-shock 70kDa protein 6
<b>7</b>	<i>HSPA7</i>	HSPA7	Heat-shock 70kDa protein 7
<b>8</b>	<i>HSPA8</i>	HSPA8	HSC70; HSC71; HSP71; HSP73
<b>9</b>	<i>HSPA9</i>	HSPA9	GRP75; HSPA9B; MOT; MOT2
<b>10</b>	<i>HSPA12A</i>	HSPA12A	FLJ13874; KIAA0417
<b>11</b>	<i>HSPA12B</i>	HSPA12B	RP23-32L15.1; 2700081N06Rik
<b>12</b>	<i>HSPA13<sup>b</sup></i>	HSPA13	Stch
<b>13</b>	<i>HSPA14</i>	HSPA14	HSP70-4; HSP70L1
<b>HSPC</b>			
<b>1</b>	<i>HSPC1<math>\alpha</math></i>	HSPC1	Hsp89, Hsp90, HSP90a
<b>HSPH</b>			
<b>2</b>	<i>HSPH2<sup>b</sup></i>	HSPH2	HSPA4; APG-2; HSP110

Adapted from (Kampinga et al. 2009)

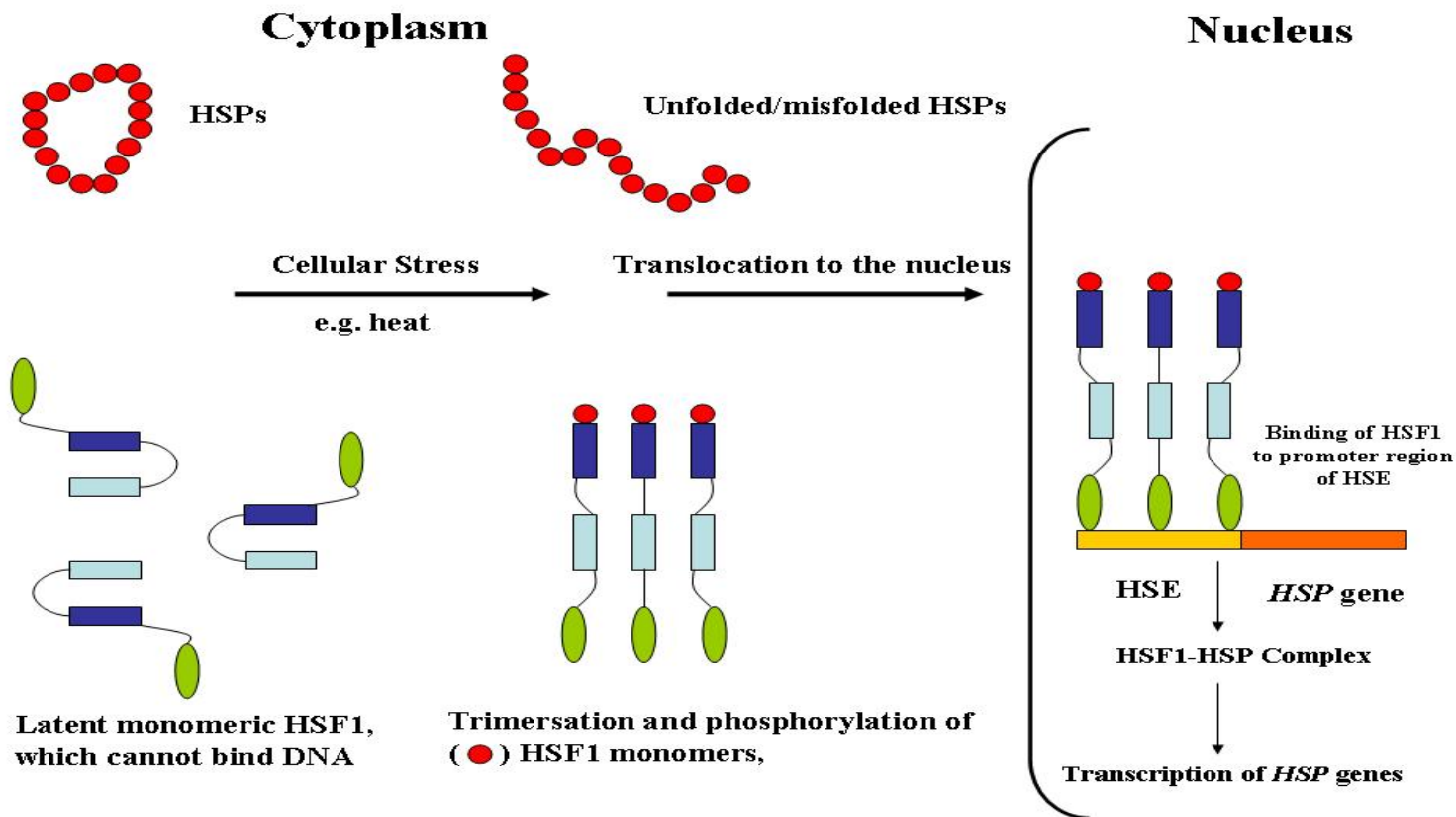


### 1.2.2 Regulation of the heat shock response

The induction of HSPs in response to various stressors has to be tightly regulated at the transcriptional, translational and post-translational level. Transcriptional regulation is dependent upon the activation of specific members of a family of transcription factors, the heat shock factors that bind to the heat shock element (HSE) in the promoter region of *HSP* genes (Wu, 1995; Cotto, Kline & Morimoto, 1996). There are four heat shock factors (HSF 1-4) and HSF1 and HSF3 are the heat shock factors that regulate stress-induced HSP gene expression (also known as HSPB1) (Samali et al. 1999b; Soti & Csermely, 2007), however the threshold temperature required for their activation is different, with HSF1 being activated by a less severe temperature. The remaining HSFs are involved in regulating HSPs under non-stressful conditions, crucial for normal cellular physiological processes such as embryonic development and cellular differentiation (He et al. 2003), and their levels are regulated in response to a wide variety of biological processes such as immune activation.

In unstressed cells, HSF1 is present in the cytosol and kept as a latent monomeric molecule that has no deoxyribonucleic acid (DNA) binding activity through its interactions with HSPs including Hsp40, Hsp70 and Hsp90 (Soti & Csermely, 2007). However, when exposed to cellular stress HSPs dissociate from HSF1 and bind to intracellular misfolded proteins which titrate out HSF1 forming the inhibitory chaperone complex (Zuo, Rungger & Voellmy, 1995). This results in trimerisation of HSF1 by mitogen-activated protein kinases (glycogen synthase kinase 3  $\beta$  and c-jun N-terminal kinase), becomes phosphorylated to fully acquire the trans-activation capacity and is translocated from the cytosol to the nucleus. Here in this state, HSF1 trimers bind to the HSE, that is, specific cis-acting DNA sequences of *HSP* genes, activates transcription of *HSP* genes and leads to elevated levels of HSPs and the formation of HSF1-HSP complexes (Zuo et al. 1995). Finally, as the cellular stress is reduced, the trimeric forms of HSF1 dissociate from the DNA (HSE) and are converted back into HSF1 monomers. Furthermore, it has been proposed that inducible HSPs themselves may negatively regulate HSF1, through re-localising to the nucleus, where they bind to the HSF1 trans-activation domain and repress transcription of *HSP* genes (Shi, Mosser & Morimoto, 1998). The primary regulator of HSF1 is Hsp90, which maintains HSF1 in its inactive, compacted form in the cell (Zuo et al. 1998).

Inhibitors of Hsp90, such as geldanamycin can inhibit all steps of the stress response, indicating the central role of Hsp90 (Zuo et al. 1998). The regulation of the heat shock response by HSF1 refers to members of the HSP27 and HSP70 families *via* negative control of HSF1 and destabilisation at the messenger ribonucleic acid (mRNA) level. Regulation of the heat shock response is summarised in figure 1.1.



**Figure 1.1: Induction and regulation of the heat shock response.**

Under normal conditions, HSF1 exists as a latent monomer in the cytosol. Upon exposure to cellular stress such as heat shock, HSF1 monomers form trimers, are phosphorylated and migrate to the nucleus. In the trimeric state, HSF1 binds to the promoter region of the HSE of *HSP* genes, forming a HSF1-HSP complex leading to induction of *HSP* gene transcription. (Adapted from Pockley, 2003a).

### **1.3 Heat Shock Protein 27**

Hsp27 is an important member of the small, low molecular weight HSP family (molecular weight 27 000 in humans and 25 000 in rodents), which is ubiquitously expressed in human cells, both in normal cells (breast, uterus, cervix and skin) and cancerous cells (tumours of the breast, liver and prostate), at specific stages of development and differentiation (Arrigo et al. 1988). However, the expression levels seem to vary with some cells expressing relatively low levels while other cells express Hsp27 abundantly. Its expression is also associated with several other human diseases, such as atherosclerosis (Xu et al. 2002; Wick et al. 2004; Rayner et al. 2008), AD (Renkawek, Bosman & de Jong, 1994), and Parkinson's disease (PD) (Renkawek, Bosman & Giel, 1999). Hsp27 is predominantly an intracellular protein (Feder & Hofmann, 1999), which is constitutively expressed in non-stressed cells, usually existing as a large oligomeric unit of up to 800 kDa mainly within the cytosol but also in the perinuclear region in the detergent-soluble fraction near the Golgi complex (Arrigo et al. 1988). Similar to Hsp72, Hsp27 is a stress-inducible protein and its synthesis and rapid phosphorylation is induced by heat shock and other environmental and pathophysiological stressors such as oxidative stress, UV radiation, and ischaemia (Arrigo et al. 1988; Arrigo et al. 2004). In response to heat shock, Hsp27 is localised within the nucleus in the detergent-insoluble fraction (Arrigo et al. 1988), whilst other stressors for example sodium arsenate distribute the protein into both the soluble and insoluble fractions (Arrigo et al. 1988). Thus, the intracellular localisation of Hsp27 appears to change according to the physiologic state of the cells and the type and intensity of the stressors. The reason for such variability is thought to be due to the fact that Hsp27 can oligomerize (regulated by phosphorylation), into large aggregates up to 1000 kDa in size, which are then insoluble in the presence of detergents (Behlke et al. 1991).

Hsp27 is a multifunctional protein that participates in several processes inside cells. Hsp27 acts as an ATP-independent molecular chaperone in protein folding (Jakob et al. 1993; Vos et al. 2008), but is also implicated in thermotolerance, survival, and recovery of cells when exposed to stressful conditions, through its capability to bind and stabilise the actin cytoskeleton preventing irreversible protein aggregation and ensuring refolding of misfolded polypeptides and

proteins by Hsp70. In addition to its chaperone function, Hsp27 also regulates structural integrity and membrane stability, cell growth/differentiation, cell migration, inhibition of apoptosis and tumour progression (Arrigo et al. 2002; Kostenko, Johnnessen & Moens, 2009). Several reports also indicate that Hsp27 may be involved in signal transduction processes of cell regulators. Indeed Hsp27 has been shown to play a role in the activation of the signal transduction pathway leading to monocyte production of the anti-inflammatory and immunoinhibitory cytokines interleukin-10 (IL-10) (De et al. 2000), macrophage colony-stimulating factor (M-CSF) (Hashimoto et al. 1997), and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (Hwang, 2000). De et al. (2000) found exposure of human monocytes to Hsp27 induces the production of the anti-inflammatory cytokine IL-10, which can inhibit the production of other pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF- $\alpha$ ), suggesting Hsp27 may also have anti-inflammatory functions and participate in inflammatory control.

#### **1.4 Heat shock protein 60**

Hsp60 also known as chaperonin 60 (Cpn60), belongs to one of the most prominent and well characterised molecular chaperone 60 kDa families, which are present in many species, and include members such as plant Hsp60, otherwise known as rubisco-binding protein; GroEL, the *E. coli* Hsp60 and Hsp65, a major antigen of mycobacteria (Craig, Gambill & Nelson, 1993). Members of the HSP60 family are abundantly expressed, accounting for 15-30 % of all cellular proteins in cells under control conditions and are induced to a higher concentration in response to various stressors (Craig et al. 1993; Hartl, 1996). In unstressed eukaryotic organisms, mammalian Hsp60 is present in organelles, predominantly within the mitochondria and to varying degrees in other cellular compartments including the cytosol, whilst plant Hsp60 is located in chloroplasts (Gupta, 1995; Hartl, 1996). The organellar Hsp60s exhibit a very high sequence homology to GroEL, with more than half of the residues being identical, and are therefore likely to have originated from bacteria belonging to the proteobacteria and cyanobacteria groups (Gupta, 1995; Gray, 1999). Interestingly, antibodies to mammalian Hsp60 are also present in isolated erythrocytes, which appears surprising since these are enucleate cells (Alberts et

al. 2002). In response to stress, apoptotic conditions or inflammation, synthesis of Hsp60 is rapidly up-regulated in microbial as well as eukaryotic cells and similar to Hsp27, changes in intracellular localisation are observed, including some expression on the cell surface (Wand-Württenberger et al. 1991; Soltys et al. 1997). There is accumulating evidence for the involvement of Hsp60 in the pathology of several human diseases and disorders, for example microbial Hsp60 is found to be a major target of the immune defence to infection (Fernandez et al. 1996; Burian et al. 2001). Furthermore, increased local expression of mammalian Hsp60 in target tissues has been found in various chronic inflammatory conditions, including RA (Brudzynski, Martinez & Gupta, 1992), insulinitis (Holoshitz et al. 1986), atherosclerosis (Xu et al. 1993; Xu et al. 2000; Pockley et al. 2000; Wick et al. 2004) and type-I diabetes, in which anti-Hsp60 mediates hyperglycaemia (Abulafia-Lipid et al. 1999; Lai et al. 2007).

Due to the fact that members of the HSP60 family are present in many different species, it has been recognised that these proteins carry out some of the most important biological functions, including that of intracellular chaperones similar to Hsp27 (Bakau & Horwich, 1998); however these are ATP-dependent. Hsp60 with its co-chaperone Hsp10 facilitates the synthesis, maintenance and transportation of mitochondrial proteins from the cytosol into the mitochondrial matrix and assists the degradation of aged or damaged proteins *via* the proteasome (Becker & Craig, 1994; Hartl, 1996; Bukau & Horwich, 1998). In addition to these functions, Hsp60 has also been identified as having an important role in immune activation and recognition, primarily by inducing inflammatory responses in innate immune cells. In fact, it has been suggested as a target for the immune response during chronic inflammation such as atherosclerosis (Wick et al. 2004; Xu et al. 2000), and autoimmune T-cells in healthy individuals, which can in turn regulate inflammatory or autoaggressive immune reactivities (van Eden et al. 1998). Furthermore, Hsp60 has been suggested to induce regulatory T-cell responses *via* the interaction with the innate immune system (van Eden et al. 2005), indicating that Hsp60 may serve as a potential ‘danger signal’ to the innate immune system.

### 1.5 Heat Shock Protein 70

The 70 kDa HSP family (HSP70) constitutes one of the most prominent, ubiquitous and widely studied family of all the HSPs. In most species, including bacteria, protozoa, plants and animals there are many proteins that belong to the HSP70 family which, similar to other HSPs comprise constitutively expressed and heat or cellular stress-inducible members that show a high degree of conservation, having at least 50 % homology. Hsp70 proteins are present in compromised cells as well as in healthy cells and their increased expression has been implicated in autoimmune conditions such as RA (Martin et al. 2003). Members of the HSP70 family are localised in nearly all subcellular compartments of eukaryotic cells, including the cytoplasm, ER, mitochondria and chloroplasts (Boorstein et al. 1994), whilst the inducible isoforms appear to be predominantly cytoplasmic or nuclear in distribution (Tavaria et al. 1996). Within mammalian systems, the best known HSP70 family members include: the constitutively expressed cytosolic Hsc70 (Hsp73) which typically makes up 1-3 % of total cellular proteins, the stress inducible cytosolic Hsp72 up-regulated by cells in response to hyperthermia, oxidative stress, and changes in pH, the mitochondrial mtHsp70 or Grp75, and the binding immunoglobulin protein Grp78 or BiP, localised in the ER, which can also be up-regulated in response to stress or conditions of starvation (Parcellier et al. 2003). Hsp70 proteins are the major cytosolic and nuclear ATP-dependent molecular chaperones that are an important part of the cell's machinery under normal conditions and in response to conditions of cellular stress, for supporting the correct folding of nascent polypeptides and/or misfolded/unfolded proteins (Hartl, 1996; Tavaria et al. 1996). Hsp70 (DnaK in *E. coli*) is the best-characterised molecular chaperone.

As molecular chaperones, all members of the HSP70 family, regardless of location bind polypeptides and proteins, particularly unfolded ones. Hsp70 assisted folding involves repeated cycles of substrate binding and release, which their primitive structure allows for. Members of the HSP70 family contain two functionally distinct domains: the N-terminal domain and the C-terminal domain (Tavaria et al. 1996), which folds into a complex three-dimensional shape allowing the proteins to become functional in order to fulfil their intracellular chaperone roles (Meimaridou, Gooljar & Chappel, 2009). Although the overall three-dimensional structure of Hsp70 is not known, the two domains previously

mentioned have been well characterised. The N-terminal 44 kDa fragment otherwise known as the ATPase domain, has two approximately equal-sized lobed domains with a deep cleft between them in which ATP binds. This binding induces a conformational change within the C-terminal 10 kDa fragment of Hsp70 (Boorstein et al. 1994; Hartl, 1996; Lehner et al. 2004) that results in an increase in the on/off rate for a substrate polypeptide and a low binding affinity (Schmid et al. 1994). Furthermore, ATP is then hydrolysed to adenosine diphosphate (ADP) causing another conformational change leading to the conversion of Hsp70 to an ADP form that has a slow on/off rate and relatively high binding affinity for a substrate polypeptide (Schmid et al. 1994; Pierpaoli et al. 1997). As suggested, the C-terminal contains the substrate-binding domain which contains a deep pocket with specificity for hydrophobic peptides, particularly leucine. It also contains an  $\alpha$ -helical sub-domain that comprises five helices which act as a lid for the substrate binding domain (Hartl, 1996; Lehner et al. 2004).

Hsp70 proteins constitutively present inside the cell are crucial in multiple 'normal' cellular physiological processes, including protein synthesis and protein transport across intracellular membranes to the mitochondria and the ER, by stabilising them in a partially-folded state (Boorstein et al. 1994; Tavarria et al. 1996). However, primarily they facilitate folding, oligomerization and assembly of nascent polypeptides and proteins, preventing them from aggregating modulated by the presence and hydrolysis of ATP (Morimoto & Santoro, 1998; Bukau et al. 2000; Bukau, Weissman & Horwich, 2006). ATP bound to the N-terminal of Hsp70 promotes this protein to recognise and bind as monomers to small hydrophobic regions of nascent polypeptide chains as soon as they exit the ribosome (Meimaridou et al. 2009), shielding hydrophobic amino acid residues from unwanted interactions (Bukau et al. 2000; Bukau et al. 2006). Following such bindings, hydrolysis of ATP into ADP occurs, stabilising the peptides within the substrate binding domain, keeping them in a folding competent state through preventing incorrect folding until sufficient protein has been synthesised (Tavarria et al. 1996). The chaperone ATPase activity of Hsp70 proteins are aided by their co-chaperones Hsp40 and Hsp90 proteins in eukaryotes and DnaJ in prokaryotes, which also bind to the N-terminal of Hsp70 (Mayer & Bukau, 2005). Hsp70 has a high affinity for unfolded proteins when bound to ADP, and



a low affinity when bound to ATP (Mayer & Bukau, 2005). These co-chaperones dramatically increase the ATPase activity of Hsp70 in the presence of interacting peptides and allow Hsp70 proteins to release the bound proteins, pass them on to another co-chaperone and transport them to various organelles (Hartl, 1996).

When exposed to stressful stimuli, most notably heat shock, a rapid and substantial increase in the synthesis of intracellular Hsp72 occurs through nuclear factor kappa-beta (NF- $\kappa$ B) signalling and HSF1 up-regulation, which can act to protect cells. Stress normally damages proteins, causing partial unfolding and possibly aggregation. Through Hsp72 temporarily binding to hydrophobic residues of denatured proteins exposed by stress, Hsp72 can prevent aggregation and promote their rapid refolding or proteolytic degradation if the unfolding was irreversible (Nollen et al. 1999), and in turn they protect the cells nucleus, nucleolus and cytoskeleton (Welch & Feramisco, 1984). In addition to improving overall protein integrity, elevated levels of intracellular Hsp72 have also been shown to play an important role in controlling multiple signalling pathways and these interactions are thought to be related to the cell protective function of Hsp72, including directly inhibiting the process of apoptosis (Garrido et al. 2001).

## **1.6 Heat shock protein 90**

The 90 kDa HSP (Hsp90) is a member of one of the most abundantly expressed, and highly conserved families of intracellular cytosolic chaperones, the HSP90 family. It accounts for 1-2 % of all cellular proteins in most unstressed cells (Csermely et al. 1998), and similar to other HSPs can be highly up-regulated, increasing to 4-6 % of total cellular proteins under conditions of cellular stress including heat and bacteria exposure and in response to IL-6 (Csermely et al. 1998). Elevated levels of Hsp90 are also found in a number of disease states, including patients with systemic lupus erythematosus (SLE), where its expression is correlated with elevated levels of circulating IL-6 and also with disease activity, the highest being in patients with active disease (Twomey et al. 1993), several autoimmune diseases such as type-1 diabetes (Finotti & Pagetta, 2004), and also in malignant cells including breast cancer (Pick et al. 2007). Members of the HSP90 family are ubiquitous and constitutively expressed in a variety of different organisms from bacteria to mammals and include the

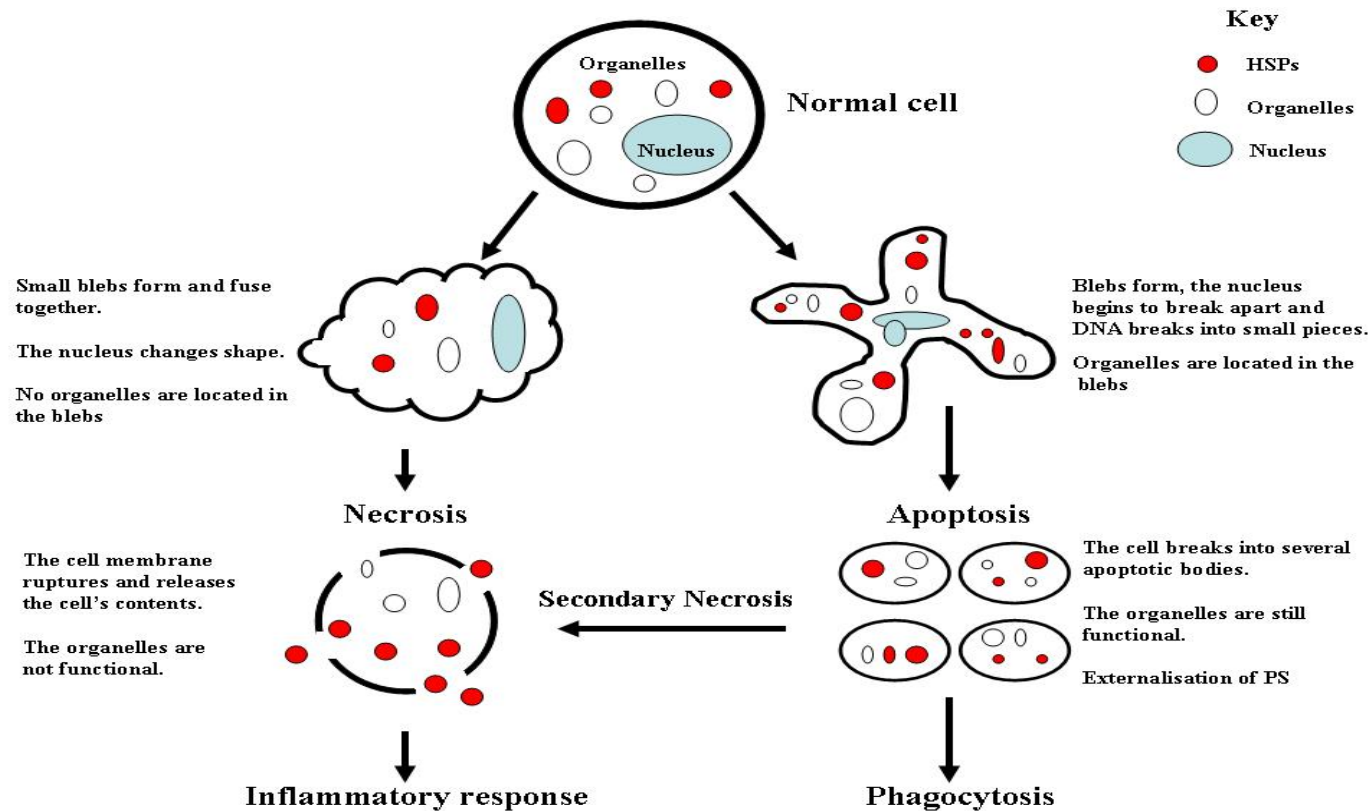
cytoplasmic Hsp90 (Hsp90-alpha/beta), ER Gp96 (grp94), and mitochondrial TRAP1 localised members (Singh-Jasuja et al. 2001), however it is absent in archaea (Chen et al. 2006). Structurally, Hsp90 shows a distinctive domain structure, characterized by a highly conserved N-terminal ATP-binding domain separated from a conserved C-terminal dimerization motif by a highly-acidic, flexible linker region and a medial substrate-binding domain (Prodromou et al. 1997).

The HSP90 family has a growing range of key, ATP-dependent, intracellular molecular chaperone functions in unstressed cells, that can be inhibited by small molecule Hsp90 inhibitors including ansamycin drugs, such as geldanamycin, by preventing binding and hydrolysis of ATP through occupying the N-terminal Hsp90 ATP-binding domain (Margineantu et al. 2007). In addition to preventing aggregation of a wide range of proteins and facilitating intracellular protein transport, Hsp90 proteins also assist in the regulation of signal transduction pathways, in cooperation with their co-chaperones Hsp40 and Hsp70 (Young et al. 2001). Unlike members of the HSP70 family, the HSP90 family does not generally act within the folding or maintenance of nascent polypeptide and protein chains, instead they are essentially involved in post-translational control. They interact and stabilise a multitude of client protein substrates, mostly conditionally activated and/or over-expressed signal transduction proteins, including steroid hormone receptors, intracellular protein kinases, growth factor receptors and transcription factors (Young, Moarefi & Hartl, 2001), in the late stages of folding, ready for interacting with other molecules that trigger their signalling function (Neckers & Ivy, 2003). Many Hsp90 client proteins such as p53, have been implicated in disease processes, and p53 for example is a member of the well characterized oncogenic pathway, making Hsp90 inhibitors attractive anti-cancer agents for their ability to induce apoptosis by redirecting oncoproteins toward ubiquitin-mediated proteolysis (Margineantu et al. 2007), thus Hsp90 proteins also function in facilitating tumour repression (Cid et al. 2008). Indeed, much of the current research on the HSP90 family is focused upon their immunological properties, aimed towards the ability of Hsp90 proteins to bind to peptides and present them to peptide-specific cytotoxic T cells initiating an effective anti-tumour immune response (Neckers & Ivy, 2003; Zitvogel et al. 2008). This has become the basis of a novel and very effective

cancer treatment (Neckers & Ivy, 2003). In addition, Hsp90 proteins are part of the lipopolysaccharide (LPS) receptor complex participating in the innate immune response to a key pathogen-associated molecular pattern (PAMP) (Triantafilou, Triantafilou & Dedrick, 2001a), in relation to this, the Hsp90 homologue, gp96 is involved in the folding of toll-like receptors (TLRs) (Triantafilou et al. 2001a).

## **1.7 Cell death**

Exposure of cells to cellular stress activates a survival response through the synthesis of intracellular HSPs, however if the exposure is intensified cell death will occur. There are two forms of eukaryotic cell death, apoptotic and necrotic and depending on the cell type or the intensity of the treatments one can observe apoptosis, necrosis or both processes. Generally speaking, low exposures have been reported to significantly increase the proportion of apoptotic cells whereas increasing exposures has been found to cause cell death by necrosis (Dypbukt et al. 1994; Mosser et al. 1997; Sapozhnikov et al. 1999; Vacca et al. 2004; Zhou et al. 2005). This suggests that some early events may be common to both types of cell death. Apoptosis and necrosis are biochemically and morphologically quite distinct (Eales, 1997), summarised in figure 1.2, but they can be caused by the same pathophysiological exposures, can be prevented by anti-apoptotic mechanisms and apoptotic cells can become necrotic (secondary necrosis) (Sreedhar & Csermely, 2004a).



**Figure 1.2: Structural changes of cells undergoing necrosis or apoptosis.**

Cells undergoing necrosis initially swell and their internal organelles break down. The cell membrane eventually ruptures and releases cellular debris that leads to local inflammation. During apoptosis, the cell body shrinks and the DNA in the nucleus become condensed and break up into small fragments. The cell's organelles remain intact; however the cell breaks up into several smaller bodies that are still surrounded by a membrane. These 'apoptotic bodies' are then engulfed and destroyed by scavenging cells; if they are not effectively cleared secondary necrosis will occur. (Adapted from Goodlett & Horn, 2001).

### 1.7.1 Apoptosis

Apoptosis, molecular regulated cell death (Kerr, Wyllie & Currie, 1972) is an essential physiological process and the main mechanism of cell elimination during embryogenesis, hormonal development and the maintenance of appropriate cell numbers in all multi-cellular organisms (Gabai et al. 1995; Krammer, 1999, Vaux & Korsmeyer, 1999). It is characterised by cell shrinkage, plasma membrane blebbing, loss of plasma membrane asymmetry and attachment, nuclear breakdown, chromatin condensation and the formation of apoptotic bodies, which are recognised and removed by cells of the phagocytic system (Kerr, 1971; Wyllie et al. 1980). The biochemical events that underlie these phenotypic changes include endonuclease-mediated DNA fragmentation and externalisation of PS residues (Beere & Green, 2001).

Apoptosis can be induced by a variety of signals, including the activation of Fas or TNF receptors, excessive DNA damage or cellular stress such as elevated temperature and oxidative stress (Buzzard et al. 1998). It can be divided into two distinct but interconnecting pathways. The extrinsic pathway often referred to as receptor-mediated apoptosis which is activated upon the interaction between a cell-surface death receptor (members of the TNF<sup>1</sup> receptor superfamily) and its ligand (Schneider & Tschopp, 2000). The intrinsic pathway commonly known as receptor independent apoptosis, which is initiated by cellular stress and which activates pro-apoptotic members of the Bcl-2 family to target the mitochondria (Schneider & Tschopp, 2000). This process ultimately involves the activation of a family of cysteine aspartate-specific proteases, known as interleukin-1 $\beta$ -converting enzyme (ICE)-like<sup>1</sup> proteases or caspases (Wolf & Green, 1999), which are activated in a proteolytic cascade to cleave specific substrates. There are two categories of caspases, initiator and executioner caspases. Upon receiving an apoptotic signal, initiator caspases for example, caspases-8-9-10 promote the binding of adaptor molecules to their protein-protein interaction domains, which induces the activation of effector caspases and assembly of the mature enzyme (Garrido et al. 2001). Executioner caspases for example caspases-3-6-7, processed by initiator caspases, cleave the proform of the executioner caspase allowing its assembly into the proeolytically active enzyme (Garrido et al. 2001). This process enables the cleavage of key protein substrates which cause the cellular changes associated with apoptosis.

### 1.7.1.1 Regulation of apoptosis

Apoptosis can be divided into three specific phases, the induction phase (signalling phase), the transduction phase (preparation phase) and the execution phase (death phase). Cellular stress causes a disturbance in intracellular homeostasis which activates the induction phase of apoptosis, including surface death receptors, the mitochondrial pathway or the initiation of apoptosis by other stimuli. This process is dependent upon the apoptotic signal being delivered to the target cell nucleus (Eales, 1997).

The extrinsic pathway: once activated through ligand binding, death receptors including TNF-R1/R2, CD95/APO-1/Fas, recruit adaptor polypeptides including TRADD (TNF receptor-associated death domain) or FADD (Fas-associated death domain) *via* their death domains and interact with the death effector domain of caspase-8 zymogens, subsequently activating pro-caspase-8 to cleave downstream effector caspases (Medema et al. 1997). In addition, they may trigger a signal transduction pathway which activates specific genes, including the DAXX/ASK1/JNK pathway (Arrigo & Müller, 2002). This activation results in the formation of the death-inducing signalling complex (DISC), at the cytoplasmic death domains of ligated death receptor oligomers (Kischkel et al. 1995; Kischkel et al. 2001). Pro-caspase-8 is then processed to an active enzyme at the DISC. There are two other alternative pathways downstream of caspase-8 that can trigger apoptosis. In type I cells, such as thymocytes and fibroblasts, caspase-8 directly activates caspase-3. Whilst, in type II cells such as hepatocytes, caspase-8 cleaves the pro-apoptotic protein Bid, a member of the Bcl-2 family which activates pro-apoptotic molecules Bax and Bak to permeabilize the mitochondria, stimulating the release of cytochrome *c* and initiating the intrinsic pathway (Arrigo & Müller, 2002).

The intrinsic pathway: once activated translocation of pro-apoptotic factors including Bax and Bak molecules from the cytoplasm to the inter-membrane space of the mitochondria controlled by Bcl-2 proteins occurs (Zimmermann et al. 2001). This causes permeabilization of the mitochondria inducing the release of many apoptogenic factors including cytochrome *c*, the flavoprotein apoptosis inducing factor (AIF) and a second mitochondria derived activator of caspase

(Smac/DIABLO) (Zimmermann et al. 2001; Ashe & Berry, 2003). The release of cytosolic cytochrome *c* triggers the formation of a multimeric complex called the apoptosome, containing apoptotic protease-activating factor 1 (Apaf-1), a mammalian CED-4 homologue and pro-caspase-9 (Zou et al. 1999). During the signal transduction phase, initiator caspases (caspases 8-10) are activated in a dATP/ATP-dependent manner which triggers the proteolytic maturation of downstream effector pro-caspases such as pro-caspase-3, initiating the caspase activation cascade (Li et al. 1997). This is followed by cleavage of apoptotic substances, making the final execution phase. However, it is not certain whether active caspase-3 is then released into the cytosol or whether it functions within the apoptosome complex (Cain et al. 1999). During the execution phase, important cellular processes are disrupted and changes in the cellular morphology occur and finally cell death, involving phagocytosis and degradation of apoptotic bodies by macrophages or neighbouring cells (Parcellier et al. 2003). In addition, Smac/DIABLO binds to inhibitor of apoptosis proteins (IAPs), preventing their function and assisting with the apoptosis cascade (Du et al. 2000). Whilst AIF directly translocates to the nucleus and triggers caspase-independent changes (Susin et al. 1999). Stress-induced apoptosis usually occurs through the activation of the mitochondria pathway, this is particularly the case when mild oxidative or heat stresses are considered (Arrigo & Müller, 2002). The apoptotic pathways are regulated by IAP proteins which directly target the caspases (Salvesen & Dixit, 1997; Salvesen et al. 2002), c-FLIP which can either promote or inhibit caspase activation at the DISC (Thome & Tschopp, 2001) or Bcl-2 proteins which target the mitochondria (Cory & Adams, 2002). Furthermore, the extrinsic pathway can be regulated by the relative expression of death and decoy receptors (Ashkenazi, 2002).

Malfunctions in the control of apoptosis have been implicated in several human diseases such as cancer (Trieb et al. 2000) and neurodegenerative disorders including AD (Tiraboschi et al. 2004).

### **1.7.1.2 Apoptosis and heat shock proteins**

Because of the implicated roles of apoptosis in several human diseases and disorders it is of significant importance to find a way to limit apoptosis and its effects. Several proteins have been suggested as therapeutic agents to inhibit

apoptosis, including members of the Bcl-2 family by preventing the release of cytochrome *c* (Salvesen & Dixit, 1997), IAPs which block caspase-3 activation (Salvesen & Dixit, 1997), and members of the HSP family through the direct inhibition of mitochondrial membrane depolarisation, apaf-1, Bax translocation and cytochrome *c* release (Liu et al. 1996; Stankiewicz et al. 2005), as well as inhibiting caspase-3 and SAPK/JNK activation (Li et al. 2000; Mosser et al. 1997; Mosser et al. 2000; Volloch et al. 2000). HSP interaction with apoptotic pathways are summarised in figure 1.3. In addition, members of the HSP70 family have also been shown to interfere with several caspase-independent pathways where PS externalisation to the cell surface and mitochondrial alterations are observed (Creagh, Carmody & Cotter, 2000; Milleron & Bratton, 2006).

A number of anti-apoptotic functions of intracellular Hsp27 have been suggested. Ricci and co-workers (2001) demonstrated a total resistance of the human leukaemic Jurkat T cell line to Fas- and staurosporine mediated apoptosis, in response to the over-expression of Hsp27 induced by a prior heat shock at 42°C (Ricci et al. 2001), thus suggesting Hsp27 to prevent receptor-mediated apoptosis. More recently, Li et al. (2007) using a rat cardiac cell line H9c2, found the over-expression of intracellular Hsp27 conferred resistance from oxidative stress-induced apoptosis by hydrogen peroxide exposure. Hsp27 has been proposed to bind to cytochrome *c* in the cytosol, preventing formation of the apoptosome and the downstream activation of caspase-3 by caspase-9 mediated proteolysis (Pandey et al. 2000a; Garrido et al. 2001; Samali et al. 1999a; Concannon, Gorman & Samali, 2003), as well as preventing the release of cytochrome *c* and Smac/DIABLO from the mitochondria into the cytosol (Paul et al. 2002; Chauchan et al. 2003), thus preventing receptor independent apoptosis.

There are debatable studies reporting the involvement of Hsp60 in the inhibition of apoptosis. However, Lin et al. (2001) found the over-expression of Hsp60 in the cytosol of cardiac myocytes by stable transfection using an adenoviral vector, increased the survival rate of these cells undergoing ischaemia injury (Lin et al. 2001). Chandra and co-workers (2007) also found cytosolic Hsp60 to reduce staurosporine induced apoptosis in PC3 (prostate cancer) and GM701 (transformed human fibroblast) cells (Chandra, Choy & Tang, 2007). It has been



suggested that Hsp60 facilitates the removal of Bax-containing complexes (Shan et al. 2003) and stabilises IAPs (Ghosh et al. 2008) thus preventing apoptosis.

Intracellular Hsp72 has also been demonstrated to block caspase-dependant apoptosis, suppress mitochondrial damage and nuclear fragmentation (Mosser et al. 1997; Buzzard et al. 1998; Beere & Green, 2000; Lang et al. 2000; Takuma et al. 2002; Clemons et al. 2005). Wei et al. (1995) suggested abrogation of Hsp70 expression by use of antisense oligonucleotides led to the inhibition of tumour cell proliferation and apoptosis (Wei et al. 1995). Consistent with this, Lang et al. (2000) found exposure of human monocytes to heat shock or IL-4 induced a rapid and marked up-regulation of Hsp72 without evoking injury or apoptosis, suggesting that Hsp72 conferred protection and survival, through producing a transient state of thermo-resistance. Similarly, Li et al. (2000) revealed Hsp72 to inhibit apoptosis, but more specifically caspase-3 cleavage and DNA fragmentation, suggesting Hsp72 prevents apoptosis downstream of cytochrome *c* release but upstream of caspase-3 activation by binding to Apaf-1.

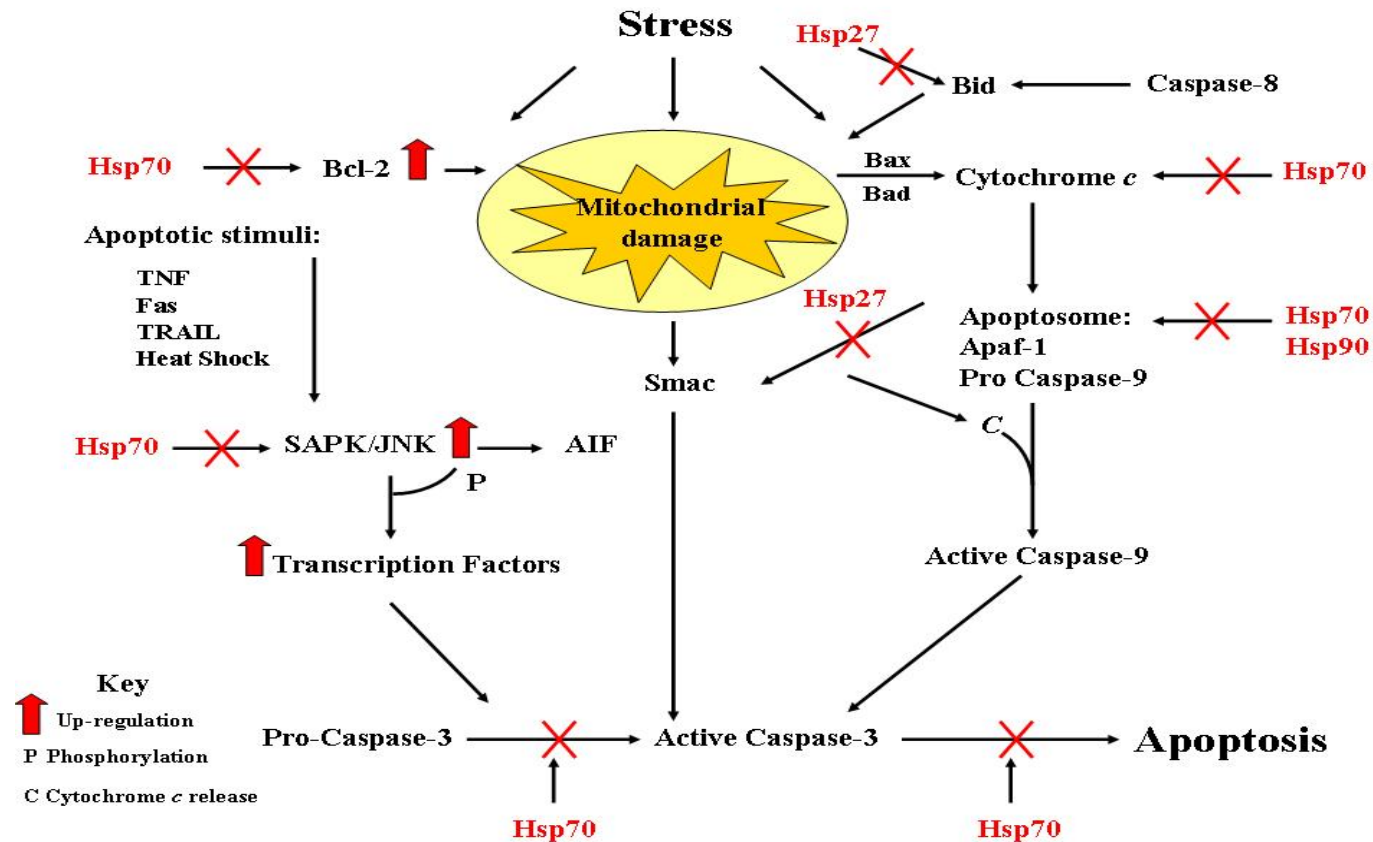
In addition, Hsp72 has been suggested to prevent caspase-9 recruitment to the apoptosome complex, thereby preventing the assembly of a functional apoptosome (Beere et al. 2000). However, in TNF- $\alpha$  induced apoptosis, Hsp72 synthesis did not prevent caspase-3 activation, instead Hsp72 inhibited late caspase-dependent events including changes in nuclear morphology, which are consistent with the characteristics of apoptotic cells and inhibited the activation of cytosolic phospholipase A<sub>2</sub> (Jäättelä et al. 1998; Saleh et al. 2000).

Hsp72 has also been proposed to act on the apoptotic pathway at an earlier step, by preventing protein kinase (SPAK/JNK) activation particularly within tumour cells (Gabai et al. 1997; Mosser et al. 1997; Buzzard et al. 1998; Volloch et al. 2000). JNK suppression was observed in cells pre-treated with a mild heat shock, indicating that Hsp72 is able to block apoptosis by inhibiting signalling events upstream of SPAK/JNK activation. However, cells in which Hsp72 was constitutively expressed, SPAK/JNK activation were not inhibited. Furthermore, JNK-independent apoptosis induced by Fas cannot be suppressed by Hsp72 (Gabai et al. 1997), implying that JNK activity could be a target of Hsp72. Hsp72 has also been demonstrated to rescue cells from TNF- $\alpha$  induced apoptosis, downstream of JNK activation, revealing Hsp72 may prevent the effector step of apoptotic cell death (Mosser et al. 1997; Jäättelä et al. 1998). Unlike the well-

characterised anti-apoptotic functions of Hsp27 and Hsp70, Hsp90 has not been proposed as a general inhibitor of apoptosis. However, in small-cell lung carcinoma it has been suggested to undertake this role (Rodina et al. 2007), through binding to many of the key proteins which regulate the intrinsic pathway of apoptosis (Neckers & Ivy, 2003).

Not all studies support the notion that HSPs offer cyto-protection, in fact over-expression of Hsp70 was found to enhance apoptosis in acute myeloid leukaemia cells (Chant et al. 1996) and Fas-mediated apoptosis in Jurkat cells (Liossis et al. 1997). Not only has Hsp70 expression been implicated in potentiating apoptosis but the high expression of intracellular Hsp90 in monocytic U937 cells has also been associated with increased apoptosis after TNF- $\alpha$  treatment (Galea-Lauri et al. 1996). Similarly, intracellular Hsp60 has been referred to as a pro-apoptotic protein enhancing the activation of caspases (Samali et al. 1999a; Xanthoudakis et al. 1999). It was found for example, that Hsp60 complexes with pro-caspase-3 accelerated the maturation of pro-caspase-3 by upstream activator caspases during apoptosis induced by camptothecin in HeLa and Jurkat cells (Samali et al. 1999a; Xanthoudakis et al. 1999).

Extracellular HSPs have also been implicated in the process of apoptosis, in particular Hsp72. This protein has been shown to render U937 monocytes and neuroblastoma cells more resistant to TNF- $\alpha$  and staurosporine-induced apoptosis (Guzhova et al. 1998; Guzhova et al. 2001). In contrast, the release of Hsp70 from human carcinoma cells induced by an inhibitor of phospholipase C activity, led to a concomitant reduction in intracellular levels of Hsp70 which left cells more sensitive to apoptotic effects of hydrogen peroxide (Evdonin et al. 2004).



**Figure 1.3: Modulation of intracellular apoptotic pathways by HSPs.**

At the mitochondrial level, Hsp27 through Bid and Hsp70 by inhibiting Bax can inhibit the mitochondrial release of pro-apoptotic proteins. After the mitochondrial stage, Hsp27 binds to cytochrome c, Hsp70 and Hsp90 bind to Apaf-1 resulting in the inhibition of apoptosome function and prevention of caspase activation and subsequently apoptosis. Hsp70 can also bind to JNK1 resulting in inhibition of JNK activation. (Adapted from Garrido et al. 2001; Parcellier et al. 2003).

### 1.7.2 Necrosis

Necrosis is the death of living cells and tissues (Kerr, 1971; Lesit & Nicotera, 1997), resulting from acute cellular dysfunction induced by external factors such as infection, toxins or trauma (Njemini et al. 2003; Moehler et al. 2003; Pittet et al. 2002). In contrast to apoptosis, necrosis is a passive process, characterised by deficient generation of ATP, swelling of organelles, rapid loss of membrane integrity and the uncontrolled release of inflammatory cellular contents, such as HMGB1 into the extracellular milieu, which subsequently leads to inflammation (Fink & Cookson, 2005).

#### 1.7.2.1 Necrosis and heat shock proteins

There is a limit to the literature reporting the involvement of HSPs in necrosis. Despite this, it has been suggested that the up-regulation of intracellular HSP provides the protection of certain cells from necrosis. These include heat shock induced expression of Hsp72 and Hsp90 in cultured neurones from glutamate neurotoxicity *in vitro* (Rordorf, Koroshetz & Bonventre, 1991), heat shock induced Hsp72 in Ehrlich ascites carcinoma cells from ischaemic necrosis (Gabai & Kabakov, 1993), moderate hypothermia induced Hsp72 from ischaemic neuronal injury (Kumar et al. 1995), supraphysiologic stress induced Hsp32 from ischaemic-induced tissue necrosis *in vivo* (Harder et al. 2005), and over-expressed Hsp72 in rat chondrocytes from experimental osteoarthritis (Grossin et al. 2006).

The mechanisms by which HSPs are able to offer protection from necrosis are not fully elucidated, however previous findings demonstrate some signalling molecules thought to be specific for apoptosis, including caspase-8-10 participate in necrosis (Leist & Nicotera, 1997). HSPs may therefore function in inhibiting caspase activation during necrosis, a mechanism involved in the inhibition of apoptosis. Consistent with this theory, Leist and Nicotera (1997) found CD95 induced necrosis in ATP-depleted cells to be repressed by inhibitors of caspases. Therefore, the effector molecules actively involved upstream in the death programme may have a role in both modes of cell death.

### **1.8 Release of heat shock proteins**

It is well established that HSPs inhabit intracellular locales, however since the mid 1980s evidence has emerged suggesting these proteins can associate to the cell membrane (Multhoff et al. 1995), and might be secreted through an unknown mechanism into the extracellular milieu (Tytell et al. 1986; Hightower & Guidon, 1989). Furthermore, it was also discovered that HSPs such as Hsp60 and Hsp70 are found in the peripheral circulation of healthy individuals in the absence of a pathological event (Pockley et al. 1998; Pockley et al. 1999; Wright et al. 2000; Lewthwaite et al. 2000a), and in response to conditions of pathological or biological stress, including injury (Pittet et al. 2002), surgery (Dybdahl et al. 2002; Dybdahl et al. 2004), and infection (Njemini et al. 2003; Moehler et al. 2003). Hsp70 release has been reported from cultured rat embryo cells (Hightower & Guidon, 1989), glial cells (Guzhova et al. 2001) and B-lymphoblastoid cell lines in response to heat shock (Clayton et al. 2005), cultured human carcinoma cells following treatment with drugs acting on protein trafficking or the phospholipase C inhibitor, U73122 (Broquet et al. 2003; Evdonin et al. 2004), and Fleshner et al. (2004) found psychological stress induced by exposure of a Sprague Dawley rat to a cat resulted in significant release of Hsp72 into the circulation when measured two hours post exposure (Fleshner et al. 2004). Hsp70 is not the only molecular chaperone that is released from cells. Previously, Hsp27 release was demonstrated from human macrophages and aortic endothelial cells treated in culture with 17 $\beta$ -estradiol (Rayner et al. 2008). Hsp60 was found within synaptophysin-containing microvesicles released from insulin-secreting  $\beta$ -cells (Brudzynski et al. 1993), and also found in human serum and conditioned media of cell cultures from glial and neuroblastoma cells following a mild temperature elevation or exposure to neuropeptide vasoactive intestinal peptide (VIP) (Bassan et al. 1998). Hsp90 $\alpha$  release has been reported from cultured vascular smooth muscle cells in response to oxidative stress (Liao et al. 2000) however, this release appears to be selective, because Hsp90 $\beta$  is not secreted under such conditions (Liao et al. 2000). These studies suggest HSP release is widespread amongst several cell types, implicating their potential roles in many physiological and pathological events. However, some important questions remain, how are HSPs transported from the cytosol to the plasma membrane and how do they reach the extracellular

environment? Currently two mechanisms are recognised: a passive release mechanism and an active release mechanism which are summarised in figure 1.4.

### **1.8.1 Passive release mechanism**

Several laboratories have reported HSPs including gp96, calreticulin, Hsp90 and Hsp72 released from stressed, viral-infected (Moehler et al. 2003) or necrosis-induced cells (Basu et al. 2000b; Saito et al. 2005), but not by cells undergoing apoptosis (Melcher et al. 1998; Gallucci et al. 1999; Basu et al. 2000b). Following severe trauma, necrotic cell death results in the discharge of intracellular contents into the extracellular milieu, clearly contributing to the release of HSPs. Pittet et al. (2002) found significant levels of Hsp72 in circulating serum of severely traumatised patients, whilst Laudanski and co-workers (2005) found significant levels of Hsp27 in circulating serum of severely injured patients following trauma (Laudanski, De & Miller-Graziano, 2005). The survival of those patients and/or the severity of the post injury inflammatory response were correlated with the levels of serum Hsp27 and Hsp72. Yet, the most conclusive evidence that necrosis causes HSP release is following the infection of cells with a variety of microorganisms such as lytic viruses (Moehler et al. 2003; O'Shea et al. 2005). Moehler et al. (2003) found infection of SK29-Mel-1 cells with the lytic parvovirus H1 was accompanied by a strong release of Hsp72, which was demonstrated to be higher and of longer duration when compared with the classical non-lethal heat shock treatment.

### **1.8.2 Active release mechanism**

One of the earliest investigations demonstrating HSP release was Tytell et al. (1986) who reported the transfer of glia-axon transfer proteins (Hsp70 and Hsc70) from adjacent glia cells into the squid giant axon, prompting the suggestion that glia cells could protect adjacent neuronal cells that were deficient in the response to stress. Consequently several years later, Guzhova and co-workers (2001) demonstrated the release of Hsp70 by T98G human glioma cells and the uptake by LA-N-5 human neuroblastoma cells under both basal and heat-stressed conditions (Guzhova et al. 2001). These events occur in the absence of necrotic cell death, suggesting the release of HSPs to be an active rather than a passive process. However, these studies did not address the mechanism of release

and whether HSPs are secreted as soluble proteins or whether extracellular HSPs are bound to secretory vesicles. Thus current studies are attempting to address this issue in terms of classical or non-classical secretory pathways.

The classical pathway of release allows for soluble proteins containing an N-terminal signal to be transported to the translocation apparatus of the ER. From here they are transported to the Golgi apparatus where they may undergo post-translocational modifications such as N-linked glycosylation and formation of disulphide bridges before being enclosed within secretory vesicles. The vesicles then fuse to the plasma membrane, leading to release of proteins and interaction with the extracellular milieu (Nickel, 2003). However, this release has been shown to be un-affected by the pharmacological inhibitors of the classical secretory pathway (ER/Golgi transport), monensin and brefeldin A (Hightower & Guidon, 1989; Broquet et al. 2003; Hunter-Lavin et al. 2004b; Lancaster & Febbraio, 2005). This supports the theory that HSP secretion may follow non-classical export routes. However, studies by Evdonin and co-workers have shown that the pharmacological inhibitor of phospholipase C, U73122 induces the release of Hsp70 from the A431 human squamous carcinoma cell line (Evdonin et al. 2004) and human keratinocyte cells (Evdonin et al. 2006). These studies suggest inhibiting phospholipase C activity as a possible mechanism of HSP release from cells, in addition to suggesting the release occurs *via* vesicular transport. Indeed, they identified chromogranin A (CGA) positive secretory-like granules located at the cell periphery as mediators of Hsp70 release, which was sufficiently blocked with an inhibitor of conventional exocytosis, brefeldin A treatment (Evdonin et al. 2004). However no lipid bodies, exosomes or lysosomes were identified, eliminating these as possible mechanisms of release and suggesting the release to be dependent on the common secretory pathway. In addition, Hsp60 has also been identified in secretory granules in cells from pituitary gland and pancreas (Cechetto et al. 2000), suggesting Hsp60 to be also released in this way. Several laboratories however disagree with this theory because HSPs do not have a peptide leader sequence that would target them to secretory vesicles for classical release and the investigations by Evdonin and co-workers did not address the issue of how Hsp70 became concentrated in secretory vesicles.

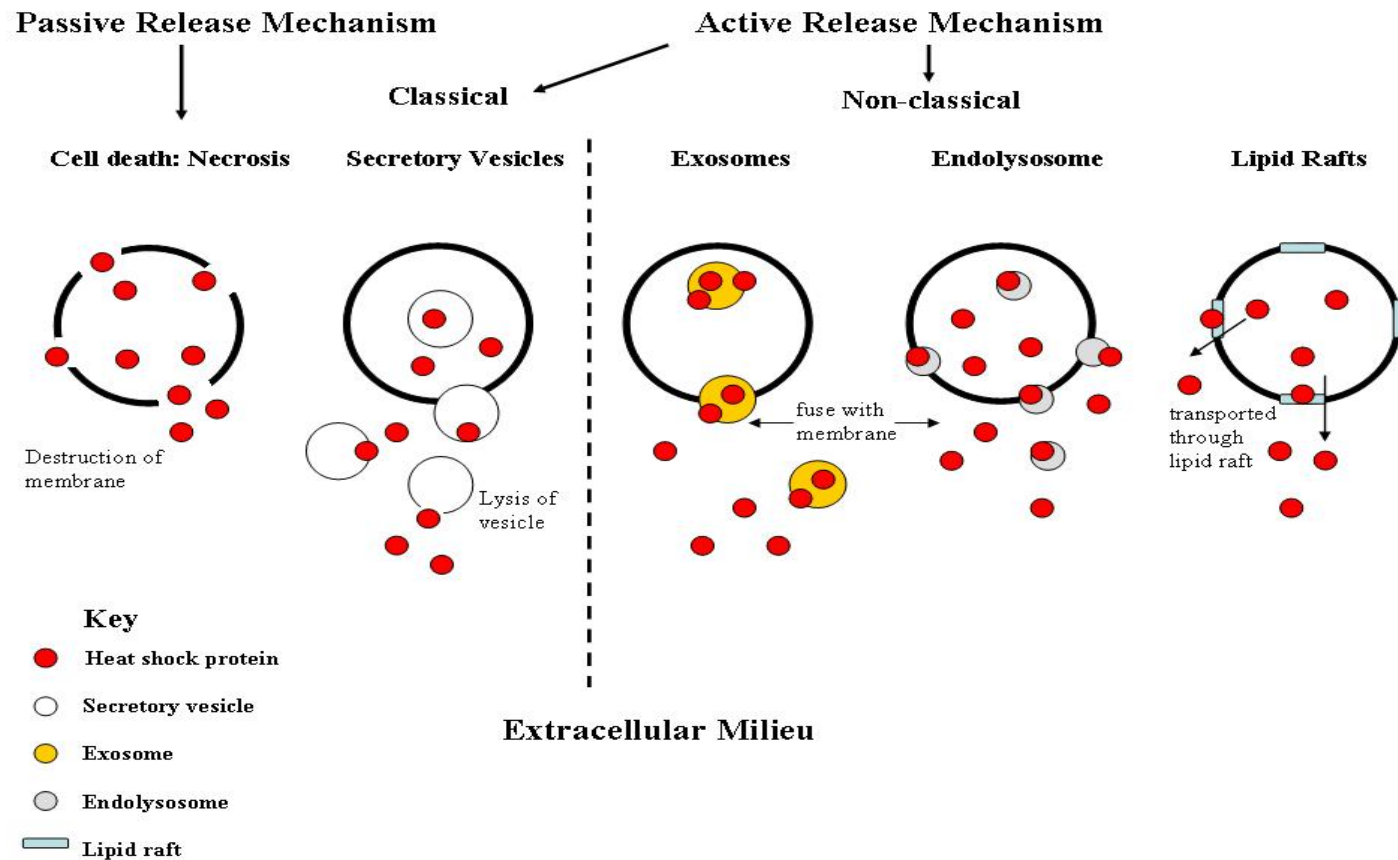
More recently studies have demonstrated the release of HSPs induced by INF- $\gamma$  and IL-10 from tumours and intact cells (Barreto et al. 2003; Bausero et al. 2005) in terms of non-classical release pathways *via* exosomes (Multhoff & Hightower, 1996; Denzer et al. 2000; Clayton et al. 2005; Gastpar et al. 2005; Lancaster & Febbraio, 2005), endolysosomes (Hunter-Lavin et al. 2004b; Mambula & Calderwood, 2006a; Mambula & Calderwood, 2006b) or lipid rafts (Broquet et al. 2003; Hunter-Lavin et al. 2004b; Vega et al. 2008). Exosomes are internal 40-90 nm small membrane vesicles that form within late endocytic compartments called multivesicular bodies (MVBs), when a segment of the cell membrane is spontaneously endocytosed. They are secreted following the fusion of MVBs with the plasma membrane into the extracellular milieu (Gastpar et al. 2005), which can be enhanced by exogenous stress (Asea et al. 2001; Clayton et al. 2005). Exosomes are actively released by professional APCs including DCs (Zitvogel et al. 1998; Théry et al. 2001), B and T lymphocytes (Raposo et al. 1996; Denzer et al. 2000), reticulocytes (Dardalhon et al. 2002), and tumour cells including human pancreas and colon carcinoma sub-lines which contain Hsc70 or Hsp70 (Raposo et al. 1996; Gastpar et al. 2005). These cells differ in their ability to present HSP on their plasma membrane, for example DCs are enriched with Hsp70, Hsc70 and Hsp90 whereas exosomes released from reticulocytes contain Hsp72 (Dardalhon et al. 2002). In addition on the surface, exosomes present tetraspanning proteins and mimic the plasma membrane of tumour cells from which it was released. It has been suggested that the tumour cell surface contains similar levels of Hsp70 and Bag-4 to the exosome surface, which were found to stimulate migration of CD94<sup>+</sup> natural killer (NK) cells towards the Hsp70<sup>+</sup> tumour cells and stimulate NK cell activity (Gastpar et al. 2005; Gehrman et al. 2005).

It has also been demonstrated that released Hsp72 following cellular stress integrates into the Golgi region of HeLa cells (Schneider et al. 2002) and is concentrated in close proximity to the cellular membrane. It is therefore assumed that HSPs may associate directly with the plasma membrane lipids such as PS, as suggested by Hightower and Guidon (1989). Here HSPs interact with membrane lipids forming ion conducting channels, increasing potassium channel activity and promoting spontaneous formation of liposomes (Alder et al. 1990; Negulyaev et al. 1996; Arispe, Doh & De Maio, 2002; Schneider et al. 2002).



Such activities were found to be modulated by ADP and ATP. This was confirmed by Vega et al. (2008) who showed Hsp70 translocates into the plasma membrane following stress, integrating into the artificial lipid with a particular specificity for PS. It has been proposed that PS, a component of the cytosolic side of the plasma membrane under non-stress conditions, may facilitate the release of HSPs once bound, through spontaneous flipping to the cell surface under stress, PS may then return to the inner membrane leaving Hsp72 embedded within the plasma membrane (Vega et al. 2008). Indeed several studies have indicated that HSPs are found in cholesterol-rich microdomains (lipid rafts) (Broquet et al. 2003; Triantafilou et al. 2002a; Hunter-Lavin et al. 2004b).

Lipid rafts are thought to be involved in the localisation of HSPs to the cell surface and the secretion of HSPs into the extracellular environment (Broquet et al. 2003). They are specialised, detergent-resistant microdomains (DRMs) that are enriched in acetylated proteins, cholesterol and sphingolipids (Pike, 2004). Both Hsp70 and Hsp90 have been shown to associate with such membrane microdomains (Triantafilou et al. 2002a). Their functions include cholesterol transport, membrane sorting, transportation of intra and extracellular proteins and signal transduction (Pralle et al. 2000). Most of these functions require HSPs through their chaperoning abilities; therefore it is not surprising to find HSPs within rafts. These findings have been confirmed in the lipid rafts of unstressed and heat shocked Caco-2 cells (Broquet et al. 2003), B cells (Clayton et al. 2005), in the lipid rafts isolated from P2 membrane fraction of rat brain (Chen et al. 2005) and in macrophages, which enhanced the processing and presentation of internalised antigens (Wang et al. 2006). The transport of proteins across membranes can be disrupted with the raft-disrupting drug methyl- $\beta$ -cyclodextrin, rather than monensin and brefeldin A, ruling out the classical secretory pathway. Methyl- $\beta$ -cyclodextrin suppresses the release of Hsp72 in the cell line Caco-2 (Broquet et al. 2003) and PBMCs (Hunter-Lavin et al. 2004b) by the removal of cholesterol, an integral component of lipid rafts. Although, Lancaster and Febbraio (2005) were unable to confirm a role for lipid rafts in stress-induced Hsp72 release from PBMCs.



**Figure 1.4: Proposed mechanisms of HSP release from cells.**

HSPs are released through two proposed mechanisms: passive or active. (Adapted from Basu et al. 2000b; Evdonin et al. 2006; Gastpar et al. 2005; Mambula & Calderwood, 2006b; Hunter-Lavin et al. 2004b).

## **1.9 Surface expression of heat shock proteins**

Intracellular HSP expression is induced in situations of stress and consequently, in certain circumstances, HSPs unusually translocate to the cell membrane, either as membrane-bound (measured in this thesis using Stressgen antibodies) or membrane-embedded proteins (measured in this thesis using the cm.Hsp70.1 antibody, Multhoff et al. 1995). HSPs are found highly expressed on the surface of virally or bacterial-infected cells (Di Cesare et al. 1992; Chouchane et al. 1994), but most predominantly on human tumour cells (Multhoff et al. 1995; Botzler et al. 1996; Kleinjung et al. 2003; Korbelik et al. 2005; Steiner et al. 2006; Cid et al. 2008; Gehrmann et al. 2008). However, to date there is no conclusive evidence of the surface expression of HSPs on normal cells derived from healthy volunteers (Multhoff et al. 1995).

There are no previous reports of Hsp27 expression on the cell surface, however mycobacterial Hsp60 has been observed on the cell surface of murine and human T cells and Hsp60 has also been found on the cell surface of normal mammalian cells (Soltys & Gupta, 1999; Soltys & Gupta, 2000). Most conclusively, Hsp70 has been observed on the surface of human T-cell leukaemia virus type 1-infected cell lines (Chouchane et al. 1994), human lung carcinoma cells (Botzler et al. 1996), chronically human immunodeficiency virus (HIV)-infected lymphoma cells (Di Cesare et al. 1992), burkitt B lymphoma cells (Di Cesare et al. 1992), human Ewing's sarcomas and osteosarcoma HOS58 cells (Multhoff et al. 1995). Furthermore, chemotherapy and radiotherapy have both been reported to induce or increase surface Hsp72 expression on treated cancer cells (Gehrmann et al. 2002; Kleinjung et al. 2003). In addition, several studies have also reported the expression of Hsp90 at the cell surface of patients with lung cancer, lymphoma and melanoma (Ferrarini et al. 1992; Becker et al. 2004) as well as on human neuroblastoma cells (Cid et al. 2008).

The presence of surface expressed HSPs in normal cells is not well understood, yet they have been suggested to possess functional roles within several important processes such as cell migration and maturation. Furthermore, they may also stabilise lipid membranes through associating with long length fatty acid chains (Guidon & Hightower, 1986; Török et al. 1997) and preserve their integrity during cellular stress (Török et al. 1997; Tsvetkova et al. 2002). Cell surface expressed HSPs may also participate in the translocation of polypeptides across

these lipid membranes (Arispe & De Maio, 2000), facilitating in cation channel formation and creating functionally stable ATP-dependent cationic pathways (Negulyaev et al. 1996; Arispe & De Maio, 2000; Arispe et al. 2002; Gross et al. 2003b).

In contrast, the cell surface expression of HSPs on human tumour cells has been correlated with cancer metastasis, relapsed tumours and the migration of malignant cells (Eustace & Jay, 2004), and in turn disease progression (Tsutsumi & Neckers, 2007). In addition, it has also been associated with an increased sensitivity to CD94<sup>+</sup> NK cell-mediated cytotoxicity following cytokine stimulation (Gross et al. 2003a). Mapping of the Hsp72 sequence revealed that this cytotoxicity occurred through the external recognition site for NK cells, the 14-mer sequence of the Hsp72 peptide termed TKD: TKDNNLLGRFELSG, aa<sub>450-463</sub>, derived from the C-terminal substrate-binding domain (Botzler et al. 1996; Botzler et al. 1998; Multhoff et al. 1997; Multhoff et al. 1999; Moser et al. 2002; Multhoff 2002; Gross et al. 2003a). This suggests that Hsp72 is embedded in the cell membrane of tumour cells but not normal cells, and the TKD region of the Hsp72 protein is exposed to the extracellular milieu through extruding the plasma membrane, which can be detected using the cm.Hsp70.1 antibody and which triggers CD94<sup>+</sup> NK mediated immune responses. Indeed, Gastpar et al. (2005) found Hsp72-high expressing tumour cells to be killed significantly better by NK cells when compared with Hsp72-low expressing tumour cells and initiated the secretion of interferon-gamma (IFN- $\gamma$ ) (Multhoff et al. 1999). Furthermore, granzyme B has also been demonstrated to bind to portions of surface expressed, membrane-embedded Hsp72 on tumour cells but not normal cells (Gross et al. 2003b). This suggests the mechanism by which NK cells eliminate Hsp72 expressing tumour cells is granzyme B-dependent, through the process of caspase-mediated apoptosis (Gross et al. 2003b). This has also been demonstrated in several infectious diseases including malaria and HIV (Lehner and Anton, 2002; Wang et al. 2002).

Several other studies have also demonstrated the surface expression of HSPs in microbial as well as eukaryotic cells in response to stress or apoptotic conditions, levels of which were found to be significantly higher than those reported in viable cells (Wand-Württenberger et al. 1991; Xu et al. 1994; Poccia et al. 1996; Soltys & Gupta, 1999; Sapozhnikov et al. 1999; Sapozhnikov et al. 2002; Feng et

al. 2003; Gross et al. 2003b; Korbely et al. 2005). In aortic endothelial cells, increasing expressions of Hsp60 proteins were detected on the cell surface in response to cytokines or high temperatures (Xu et al. 1994), which was shown to make these cells susceptible to complement-dependent lysis by Hsp60-specific antibodies (Xu et al. 1994). Furthermore, the expression of surface HSPs has been found to increase through stages of early and late apoptotic death with the highest levels observed during the loss of cell membrane phospholipid asymmetry (Sapozhnikov et al. 1999; Sapozhnikov et al. 2002). These studies suggest the membrane localisation of HSPs on apoptotic cells serve as an identifying marker for phagocytic cells (Török et al. 1997), thus correlating with the elimination of stressed, transformed or infected cells, an important feature in the protection against tumour or virally infected cells (Di Cesare et al. 1992; Chouchane et al. 1994).

### **1.10 Extracellular heat shock proteins**

Today increasing evidence suggests HSPs have importance as extracellular proteins when present at novel locations. When released into the extracellular milieu, HSPs are thought to exchange between cells, interact with adjacent cells or in some cases enter the blood stream (Calderwood et al. 2007a), where they are thought to be involved in immune responses or cyto-protection under a variety of stressful conditions (Asea, 2006; Calderwood et al. 2007b; Schmitt et al. 2007).

#### **1.10.1 Extracellular functions of heat shock proteins**

For immunologists, the most interesting aspects of extracellular HSPs are the consequences for the innate and adaptive immune responses (Ménoret et al. 2002; Campisi & Fleshner, 2003a; Campisi et al. 2003b; Williams & Ireland, 2008). Extracellular HSPs have been proposed to act as ‘messengers of stress’ or ‘danger signals’ *via* cell-to-cell signalling, that is a molecule which directly communicates a message from one cell to another (Matzinger, 1994). This theory was first suggested by Matzinger (1994), who claimed cells of the innate immune system could be activated by danger signals which originated from damaged or injured cells, rather than healthy or apoptotic cells in the extracellular environment (Matzinger 1994). The signalling molecule may be attached to the

outside of a cell or maybe released from a cell and may indeed attach to another cell. This suggests that the released HSP alerts neighbouring cells/immune cells to damage and is able to initiate an immune response. It appears that HSPs are perfect molecules for danger signalling, since they are essential, abundant and are strongly up-regulated under conditions of cellular stress. Cell-to-cell signalling has been demonstrated in human PBMCs (Lewthwaite et al. 2002b), macrophages (Kol et al. 1999), endothelial and epithelial cells (Kol et al. 1999; Zhang et al. 2001) and vascular smooth muscle cells (Sasu et al. 2001).

Extracellular HSPs, particularly Hsp60 and Hsp72 stimulate the innate immune response through binding non-covalently to receptors on cells, initiating signal transduction cascades such as the NF- $\kappa$ B signalling pathway (Ménoret et al. 2002). This triggers APCs, including macrophages and DCs to stimulate the production and secretion of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-12, TNF- $\alpha$ ) (Chen et al. 1999; Kol et al. 2000; Asea et al. 2000b Basu et al. 2000b; Svensson et al. 2006), chemokines (Lehner et al. 2000) and co-stimulatory molecules (CD40, CD83, CD86) (Bausero et al. 2005) and induces DC maturation (Basu et al. 2000b; Gallucci et al. 1999; Somersan et al. 2001; Bethke et al. 2002; Flohé et al. 2003), in order to activate the adaptive immune response (Asea, 2007). Interestingly, extracellular HSPs can also stimulate anti-inflammatory cytokines (Pockley, 2003a; van Eden et al. 2005; Wang et al. 2005a). Specific receptors on APCs, including CD91 or CD14 (Basu & Srivastava, 2000a; Basu et al. 2001; Asea et al. 2000b) also allow for the efficient and rapid uptake of HSP-peptide complexes from the extracellular milieu, which activate NF- $\kappa$ B translocation into the nucleus of the APC (Basu et al. 2000b; Singh-Jasuja et al. 2001), using both Hsp60 and Hsp70 ligands, TLR-2 and TLR-4 (Asea et al. 2000b). This up-regulates genes central to the generation of an effective immune response. The activation of NF- $\kappa$ B by Hsp72 is in contrast to its intracellular function of suppression (Chen et al. 2006; Weiss et al. 2007) and may indicate the up-regulation of Hsp72 in nearby cells in response to such danger signals. Following receptor-mediated endocytosis of the HSP-peptide complexes, the peptides are re-presented to the human leukocyte antigen (HLA) presentation pathway (Singh-Jasuja et al. 2000) on major histocompatibility complex molecules (MHC) I and II either *via* an endosomal transporter-associated antigen processing (TAP) and proteasome-independent

route or a classical TAP and proteasome-dependent ER-golgi route (Singh-Jasuja et al. 2000). This process primes cytotoxic CD8<sup>+</sup> T cell responses against the peptides associated with HSPs (Singh-Jasuja et al. 2001; Multhoff, 2002).

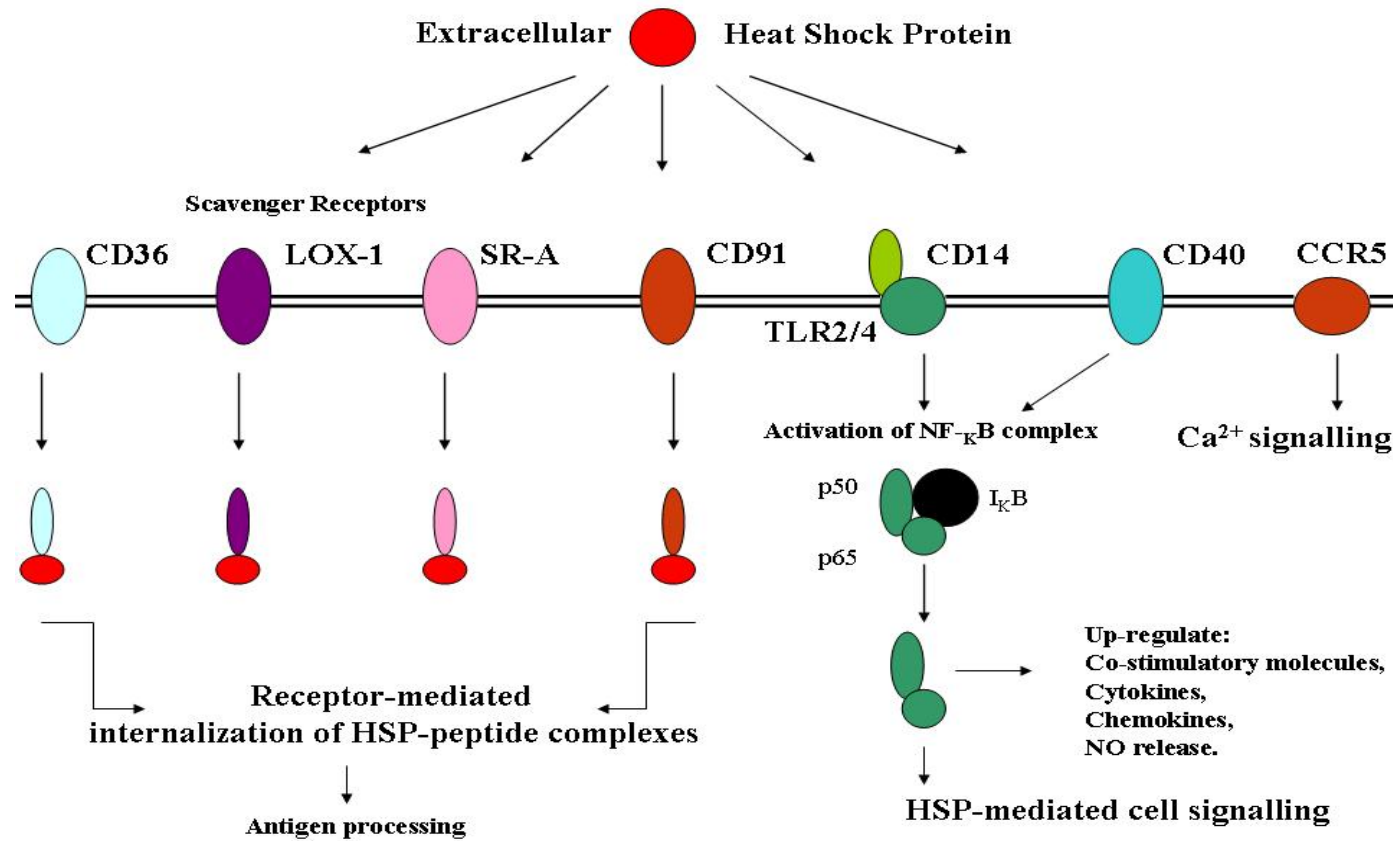
A number of studies have also considered non-immunological consequences for extracellular HSPs, such as cyto-protection with a range of cell types. Some of the earliest suggestions that HSPs may have therapeutic potential arose from the observations by Johnson and co-workers (1990) and (1993), who demonstrated exogenous Hsp72 added to the culture medium could bind to vascular-derived cells and cultured rabbit arterial smooth muscle cells and protect them from heat stress and nutrient deprivation. Several laboratories have subsequently demonstrated the cyto-protective roles of extracellular members of the HSP70 family including, spinal sensory neurons (dorsal root ganglion) from axotomy-induced death (Houenou et al. 1996; Robinson et al. 2005), cultured monocytes from TNF- $\alpha$  (Guzhova et al. 1998) and staurosporine-induced apoptosis (Guzhova et al. 2001) and skate retinal cells from degeneration (Yu et al. 2001). Extracellular Hsp27 has also been shown to protect neutrophils *in vitro* from culture-induced apoptosis (Sheth et al. 2001). However, it is not fully clear how extracellular HSPs function to protect cells. Berberian and co-workers (1990) proposed exogenous Hsp70 to increase intracellular Hsp70 levels by heat stress which was found to delay the decline in viability of stressed cells and to protect the cells from apoptotic cell death (Berberian, Johnson & Bond, 1990). Furthermore, it has been suggested that extracellular HSPs can be internalised and readily imported into cytoplasmic and nuclear compartments of many cell types, where they promote cell survival (Houenou et al. 1996; Fujihara & Nadler, 1999; Yu et al. 2001; Tidwell, Houenou & Tytell, 2004; Robinson et al. 2005).

### **1.11 Extracellular receptors for heat shock proteins**

Many of the effects of extracellular HSPs are thought to be mediated through cell-surface receptors (Calderwood et al. 2007a). When HSPs are released from cells in response to stressful stimuli they may carry intracellular derived polypeptides. Cell surface receptors may therefore recognise the HSP itself, the polypeptides or the HSP-peptide complex. However, HSPs do not bind to all cell types, indeed their binding has been described as both selective and highly specific to a number of cell types. These include APCs such as macrophages,

NKs, DCs and PBMCs (Asea, 2006). However, the question arises as to which type of receptor? Several investigations have tried to determine specific receptors that bind different HSPs on the plasma membrane and numerous have been identified. Including, SRs such as CD91, LOX-1, SR-A, CD36 and SREC-1 (Binder, Han & Srivastava, 2000; Basu et al. 2001; Delneste et al. 2002; Berwin et al. 2003; Thériault et al. 2005; Thériault et al. 2006), as well as TLRs 2 and 4 (Vabulas et al. 2001; Vabulas et al. 2002; Vabulas & Wagner, 2005), CD14 (Asea et al. 2000b), CD40 (Wang et al. 2001; Becker et al. 2002), CD94 (Gross et al. 2003a) and a stereo-specific receptor for Hsp60 (Habich et al. 2002). The binding site for Hsp60 is separate from the common receptors for Hsp70 and Hsp90 indicating an independent role of Hsp60 within immune regulation. It has also been suggested that the common HSP receptors can be divided into two groups, the first as internalising receptors for the representation of peptides, CD91 and LOX-1 and the second as signalling receptors, CD40, TLR-2 and TLR-4 (Srivastava, 2002b). A summary of potential HSP cell surface receptors are presented in figure 1.5.





**Figure 1.5: Cell surface receptors for extracellular HSPs.**

HSPs in the extracellular milieu can be recognised by an array of receptors on target cells. Cells may have one or a number of receptors. (Adapted from Calderwood et al. 2007b; Pockley, Muthana & Calderwood, 2007).

### 1.11.1 Scavenger Receptors

SRs are cell surface glycoproteins primarily expressed on APCs, endothelial and smooth muscle cells (Delneste et al. 2002; Baker et al. 1984; Pitas, 1990). They have multi-functional roles in the phagocytosis of bacteria and apoptotic cells, are involved in cell clearance of damaged cellular components and foreign substances and have a final yet well defined role in the binding and the internalisation of a broad range of ligands (Gram-positive and Gram-negative bacteria) and chemically modified low-density lipoproteins (LDL), such as oxidized LDL and acetylated LDL (Hampton et al. 1991; Armesilla et al. 1996; Gough & Gordon, 2000). Recent studies have also identified an additional role for SRs as membrane endocytic receptors, in other words binding structures for HSPs (Delneste et al. 2002). In these experiments the binding of HSPs to APCs were inhibited by molecules which bind with high affinity to SRs such as maleylated BSA (malBSA), polyinosinic acid (Poly I) and fucoidan, indicating a significant role for SRs in HSP binding to APCs (Delneste et al. 2002). SRs are categorised into eight different sub-classes (A-H) on the basis of their molecular structure (Murphy et al. 2005), yet only a select few have been identified as receptors for HSPs.

#### 1.11.1.1 CD91

The first receptor to be proposed for all immunogenic HSPs including human Hsp60, Hsp70, Hsp90, Gp96 and calreticulin was the oxidised low density lipid (oxLDL) binding protein CD91 (Binder et al. 2000). CD91 is found on the surface membranes of APCs (Binder et al. 2000; Basu et al. 2001) and is involved in receptor-mediated internalisation of various ligands and phagocytosis of bacterial toxins (Calderwood et al. 2007b). It is said to be an important protein in antigen cross-presentation in which HSPs interact with CD91 resulting in their internalisation by the receptor and the HSP-peptide complex which is critical for the induction of CD8<sup>+</sup> cytotoxic T-lymphocytes (Binder et al. 2000; Delneste et al. 2002). This interaction can be competed by  $\alpha$ 2-macroglobulin and receptor associated proteins (RAPs) (Binder et al. 2000; Basu et al. 2001; Binder & Srivastava, 2004). Indeed, Delneste et al. (2002) demonstrated *in vitro* inhibition of Hsp72 to CD91 using RAPs in human macrophages, and a weak inhibition of binding to DCs. However, its role as a direct receptor for HSPs has received

much debate, Thériault et al (2005) examined the ability of Hsp72 in free solution to bind cells with or without CD91 expression and found minimal differences, thus casting doubt on its role as a primary binder of Hsp72.

#### **1.11.1.2 LOX-1**

LOX-1, the sole member of the class E SRs is found expressed on monocytes, macrophages, immature DCs, endothelial and smooth muscle cells (Draude et al. 1999; Delneste et al. 2002). It functions as a tethering receptor for leukocyte homing and rolling on the surface of endothelial cells (Sawamura et al. 1997). LOX-1 binds with high affinity to modified lipoproteins and modified oxLDL, apoptotic cells and bacterially derived cell wall components (Gough & Gordon, 2000; Murphy et al. 2006). In addition, it has been identified as an important binding structure for human HSPs, including Hsp60, Hsp90 and especially Hsp70 within an Hsp70-peptide complex (Delneste et al. 2002; Thériault et al. 2005; Thériault et al. 2006). Through binding to LOX-1, Hsp70 is thought to be internalised by endocytosis inducing Hsp70-mediated cross-priming of CD8<sup>+</sup> cytotoxic T cells in the innate and adaptive immune response (Gough & Gordon, 2000; Calderwood et al. 2007b).

#### **1.11.1.3 SR-A**

SR-A is a class A scavenger receptor found on a number of cell types including monocytes, macrophages, smooth muscle and endothelial cells (Plüdderman, Neyen & Gordon, 1997; Gough et al. 1999). It is an important protein in cell attachment and uptake of modified lipoproteins and proteins modified by glycation and in addition form an integral part of the immune response through increasing phagocytosis of bacteria and yeast (Gough et al. 1999; Murphy et al. 2005; Lin & Karin, 2007). SR-A has been demonstrated to bind gp96 and calreticulin (Berwin et al. 2003; Thériault et al. 2005) and studies have revealed the binding of gp96 and calreticulin to macrophages to be inhibited by the polyanionic SR-A ligands fucoidan and carrageenan (Berwin et al. 2003). In addition gp96 and calreticulin bind to and are internalised by SR-A expressing HEK 293 cells but not mock-transfected cells. However, these studies did not evaluate the contamination of the gp96 preparation by LPS, as a previous study

has reported SR-A binding to LPS (Hampton et al. 1991), raising concerns for SR-A as a HSP receptor.

#### **1.11.1.4 CD36**

CD36, a member of the class B SRs is expressed on monocytes and macrophages, capillary endothelial cells, mammary secretory epithelial cells, differentiated adipocytes, B cells and erythroblasts (Talle et al. 1983; Swerlick et al. 1992; Armesilla et al. 1996; Matsumoto et al. 2000). It is implicated in adhesion, in the metabolism of long-chain fatty acids and also in the phagocytosis of apoptotic cells including neutrophils, T lymphocytes and eosinophils when expressed on macrophages (Armesilla et al. 1996; Daviet & McGregor, 1997). Here, CD36 has been reported to form part of a receptor complex (CD36- $\alpha$ V $\beta$ 3) which indeed stimulated phagocytosis (Bottchert et al. 2006). CD36 interacts with a large variety of ligands including thrombospondin, collagens type I and II, anionic phospholipids, *Plasmodium falciparum* erythrocyte membrane protein I and, similar to CD91 and LOX-1 oxLDL (Armesilla et al. 1996; Plüdderman et al. 1997). It has also been found to bind gp96, however to date it has not been demonstrated as a receptor for other HSPs (Delneste et al. 2002; Binder et al. 2004).

#### **1.11.2 Toll-Like Receptors: TLR-2 and TLR-4**

TLRs are pattern recognition receptors (PRRs) which directly recognise pathogen-associated molecular patterns (PAMPs) on microbial components (Janeway & Medzhitov, 2002). They cause NF- $\kappa$ B and interferon response factor (IRF) signalling pathway activity (Takeda, Kaisho & Akira, 2003), and stimulate the production of inflammatory cytokines and adhesion molecules which are essential for innate immunity and inflammation (Takeda et al. 2003; Calderwood et al. 2007b). There are approximately thirteen members of the TLR family which are expressed by cells of the innate immune system and each recognises different microbial components. The majority of TLRs have not been tested as HSP receptors although two TLR members, TLR-2 and TLR-4 appear to function as Hsp60, Hsp72 and Gp96 receptors (Ohashi et al. 2000; Vabulas et al. 2001). In addition, Asea et al. (2002) have demonstrated the involvement of TLR-2 and TLR-4 with CD14 activation which acts as a co-receptor for Hsp72

mediated signalling in human monocytes, inducing TNF- $\alpha$ , IL-1 $\beta$  and IL-6 production. Similarly Dybdahl et al. (2002) found Hsp72 could stimulate the production of TNF- $\alpha$  from macrophages derived from C3H/HeN mice. However, studies have doubted the interactions of HSPs with TLRs and at present it is not yet clear whether TLRs act as receptors for HSPs or are involved in signalling cellular activation. There is no evidence to indicate that there is a direct interaction between HSPs and TLRs (Thériault et al. 2005).

### **1.11.3 CD14**

CD14 is a glycosylphosphatidylinositol-anchored protein found on the surface of monocytes and macrophages and other APCs (Lehner et al. 2005). It is a receptor for the serum LPS-binding protein and LPS in conjunction with TLR-4 and MD-2 in a signalling receptor cluster (Heidenreich, 1999). CD14 was first demonstrated as a receptor for human inducible Hsp72 by Asea et al. (2000b) who found Hsp72 bound to CD14 as a result of the up-regulation of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in human monocytes. The binding involved TLR-2 and TLR-4 and the calcium-dependent activation of the NF- $\kappa$ B pathway (Asea et al. 2000b). Similarly Kol et al. (2000) characterised CD14 as a receptor for human and chlamydial Hsp60, they demonstrated that Hsp60 treatment of PBMCs and U937 cells resulted in the production of IL-6 which could be inhibited by anti-CD14. In addition, Asea et al. (2000b) also observed treatment of U373 human astrocytoma cells with Hsp70 induced the production of TNF- $\alpha$  and following transfection with CD14, the effect was enhanced (Asea et al. 2000b). This group also showed that NF- $\kappa$ B activation was similarly enhanced by Hsp70 treatment in HEK293 cells which was again enhanced following transfection with CD14 (Asea et al. 2000a). However in CHO transfected CD14 cells, Hsp72 was not found to bind to CD14 using flow cytometric analysis (Delneste et al. 2002).

### **1.11.4 CD40**

CD40 is a co-stimulatory molecule and a member of the TNF receptor superfamily found on B lymphocytes, monocytes and DCs (van Kooten & Banchereau, 1997). It plays an important role within T cell-mediated immune responses because once stimulated it binds to its counter receptor CD40L on activated T cells which causes APC activation and DC maturation (Becker et al.

2002; Millar et al. 2003), in addition to increasing CD40 expression on APCs (O'Sullivan & Thomas, 2003). CD40 has been reported as a binding receptor for microbial Hsp70 through competitive binding studies with anti-CD40 (Wang et al. 2001). This was later confirmed and extended to include murine Hsp70 in cell lysates and human Hsp70 (Becker et al. 2002) *via* the exoplasmic domain of CD40 in which experiments revealed bone marrow derived DCs require CD40 for the Hsp72 induced secretion of IL-2 (Millar et al. 2003). However, subsequent studies using CHO expressing CD40 cells failed to show the binding of Hsp70 to CD40 receptors (Thériault et al. 2005), casting debate of CD40 as Hsp70 binding receptor.

### **1.12 Extracellular heat shock proteins in health and disease**

As discussed, certain HSPs (Hsp60 and Hsp72) are present in the peripheral circulation of clinically normal individuals (>1000 ng/mL), where their presence may elicit biological effects and which are also associated with ageing (Pockley et al. 1998; Pockley et al. 1999; Rea, McNerlan & Pockley 2001; Jin et al. 2004). Their enhanced presence in the peripheral circulation and other extracellular spaces in response to several forms of cellular stresses and the immune reactivity towards them have been implicated in the pathology of several human disorders and diseases. Including, infection (Fernandez et al. 1996; Burian et al. 2001; Njemini et al. 2003), chronic inflammatory 'autoimmune' conditions such as RA (Brudzynski et al. 1992; Mapp et al. 1995; Macht et al. 2000; Martin et al. 2003), peripheral and renal vascular disease (Wright et al. 2000); cardiovascular disease (Pockley et al. 2002; Pockley et al. 2003b; Terry et al. 2004), neurodegenerative disorders (Sakahira et al. 2002; Tiraboschi et al. 2004; Merienne et al. 2003) and cancer (Trieb et al. 2000). Thus, extracellular HSPs in health and disease have become the subject of numerous experimental and clinical investigations over the past few decades.

#### **1.12.1 Ageing**

Several studies have demonstrated that there is a progressive decline of both serum Hsp60 (Rea et al. 2001) and Hsp72 levels during ageing (Jin et al. 2004), which may be due to a reduced capacity of the elderly to maintain protein homeostasis. In addition, low serum levels of HSPs have also been shown to

correlate with both spontaneous reactive oxygen species (ROS) production and increased levels of circulating pro-inflammatory markers such as TNF- $\alpha$  (Ogawa et al. 2008), suggesting that oxidative stress may also increase with age and may reflect age-associated immune dys-regulation (Ogawa et al. 2008).

Age reduces the ability of cells to respond to cellular stress, indeed it has been suggested that the heat shock response is attenuated during ageing. The activation and nuclear translocation is thought to be retained, however the binding of HSF1 to the HSE is compromised (Soti & Csermely, 2007), and this may contribute to the increased susceptibility of ageing individuals to environmental challenges, a reduced resistance to stress and increased mortality rate. Numerous *in vitro* studies have demonstrated reduced Hsp72 expression with increasing age, for example in rat hepatocytes (Heydari et al. 1995), myocardium (Gray et al. 2000), peripheral blood lymphocytes (Jin et al. 2004) and PBMCs (Richardson & Holbrook, 1996). In addition, Hsp70 gene expression has also been found to decline during normal ageing in human retina (Bernstein et al. 2000), suggesting the process of ageing maybe associated with a reduced intracellular Hsp70 production (Effros, Zhu & Walford, 1994). On the other hand, investigators have suggested an elevation of serum Hsp72 during ageing, along with an increase in pro-inflammatory markers such as serum TNF- $\alpha$  and C-reactive protein but a decrease in anti-inflammatory cytokines such as IL-10 (Njemini et al. 2007), suggesting a role for serum HSPs in inflammatory disease in the elderly (Njemini et al. 2007) and also as a prognostic marker for age-related diseases (Davidovic et al. 2007).

### 1.12.2 Infection

Elevated levels of serum Hsp72 have been detected in patients with acute infection (Njemini et al. 2003), in addition the levels of serum Hsp72 have been found to correlate with the pro-inflammatory markers IL-6 and TNF- $\alpha$ , along with the levels of the anti-inflammatory cytokine IL-10 (Njemini et al. 2003). Furthermore, microbial Hsp60 has been suggested as a major target of the immune defence to infection (Fernandez et al. 1996; Burian et al. 2001). Fever is a temporary increase in internal body temperature to levels above normal and it is a systemic response to infection (Kumar & Clark, 2002). Invading microorganisms stimulate phagocytic cells such as macrophages, to produce high

amounts of the cytokine IL-1, which activates the thermoregulatory centre in the anterior hypothalamus of the brain, stimulating fat catabolism in the brown adipose tissue for heat production. This results in an increase in the thermoregulatory set-point in the hypothalamus and this heat production finally leads to an increase of body temperature (Kumar & Clark, 2002). Oehler et al. (2001) demonstrated using human leukocytes *in vitro* and *in vivo* that the increase of body temperature during fever is able to stimulate Hsp72 expression in blood leukocytes. This presumably is a beneficial response, hypothesising the protection of leukocytes against the increased cytotoxic inflammatory mediators during inflammation, thus improving the course of the disease.

### **1.12.3 Surgery and trauma**

It has been demonstrated that surgical procedures can cause an increase in the expression of circulating HSPs. In particular, Hsp72 with the pro- and anti-inflammatory markers IL-6 and IL-10, released into the circulation following coronary artery bypass grafting (Dybdahl et al. 2002; Dybdahl et al. 2004), and in patients undergoing liver resection (Kimura et al. 2004). In these situations, circulating Hsp72 was found to be significantly associated with post-operative infection, post-operative organ dysfunction, with the inflammatory response and as an activator of the innate immune system (Kimura et al. 2004; Dybdahl et al. 2005), suggesting extracellular Hsp72 does not function to protect cells in conditions of extreme stress. However, another study has also shown that there is no relationship between Hsp72 levels and organ dysfunction (Pittet et al. 2002), or between Hsp72 levels and the severity of the post-injury inflammatory response (Pittet et al. 2002). Indeed, this study revealed the presence of increased levels of Hsp72 in the serum associated with trauma as early as thirty minutes after injury and the high levels (>15 ng/mL) were implicated with improved survival, whereas patients with low serum levels died from their traumatic injuries (Pittet et al. 2002). Furthermore, Hsp27 has also been found in the serum of severely injured patients following trauma, whereby serum levels were approximately three times higher than those found in healthy volunteers, also suggesting a role for circulating Hsp27 in inflammatory events (Laudanski et al. 2005).



#### 1.12.4 Autoimmune diseases

HSPs have been implicated in several autoimmune diseases and a number of studies have measured circulating levels of HSPs in a variety of cardiovascular disease states. Serum Hsp27 (Xu et al. 2000; Wick et al. 2004; Rayner et al. 2008) and Hsp60 levels are associated with early atherosclerosis in clinically normal individuals (Xu et al. 1992; Xu et al. 1993; Pockley et al. 1999), and with levels of the pro-inflammatory cytokine TNF- $\alpha$  (Lewthwaite et al. 2002a). In addition, elevated levels of Hsp65 antibodies are also reported in individuals over sixty years of age with carotid atherosclerosis (Xu et al. 1993) and with borderline hypertension (Frostgård et al. 1997). Elevated levels of serum Hsp72 have also been found in patients with atherosclerosis, the levels of which are used as an early marker of the disease and which can also be used to predict the progression of the disease in individuals with established hypertension. Pockley and colleagues (2003b) found patients with significantly increased levels of serum Hsp72, had a reduced thickness of their carotid intima-media (a measure of atherosclerosis), suggesting high levels of serum Hsp72 may in fact protect against or modify the progression of the disease. Terry et al. (2004) also suggested circulating serum Hsp72 as a potential biomarker of longevity. How serum Hsp72 is able to exert its protective effects in this disease is at present unclear, yet it may be due to the ability of Hsp72 to attenuate inflammatory responses by inducing Th2 CD4<sup>+</sup> T cells, which induce the anti-inflammatory cytokines IL-4 and IL-10 (Tanaka et al. 1999).

High levels of circulating HSPs also appear to be involved in the down-regulation of arthritis, of which there are two main forms, osteoarthritis and RA. RA is a systemic autoimmune inflammatory condition, characterised by synovitis and serositis (inflammation of the lining surfaces of the joints, pericardium, and pleura), rheumatoid nodules, and vasculitis (Kumar & Clark, 2002). Clinical data report serum levels of Hsp60 in patients with RA, which are found to stimulate T cells to induce anti-inflammatory cytokines, which is said to be associated with the severity of the disease (Macht et al. 2000). BiP a member of the HSP70 family is also prevalent in the joints of patients with RA (Mapp et al. 1995) and also anti-BiP antibodies are present in the serum of patients with RA. Indeed, Corrigan et al. (2001) and Blass et al. (2001) have both demonstrated BiP over-

expression in the synovial membrane of patients with RA compared with membranes from osteoarthritis patients. Thus, BiP has been implicated in the pathogenesis of the disease, for example Bodman-Smith et al. (2004) demonstrated an increasing antibody level to BiP in patients approaching the onset of the disease compared to control patients. These studies suggest anti-BiP may have a potential diagnostic role in early RA.

High concentrations of Hsp60 (Abulafia-Lipid et al. 1999; Lai et al. 2007), Hsp72 and Hsp90 have been reported in the plasma of patients with type-1 diabetes (Finotti & Pagetta, 2004). Patients with this condition typically present vascular complications by glycaemic control or by increased proteolytic activity of plasma (Finotti, Carraro & Calderan, 1992). Additionally, Hunter-Lavin et al. (2004a) have described the presence of Hsp72 in the plasma of patients with type-2 diabetes. Patients with this condition are subjected to oxidative stress as a consequence of hyperglycaemia (Sampson et al. 2002) and elevated levels of homocysteine (Stehouwer, 1999), both of which are associated with endothelial cell dysfunction (Boushey et al. 1995), and in turn cause cardiovascular complications, accounting for up to 80 % of patient deaths (Spaneimer, 2001). Treatment of type-2 diabetic patients with the anti-oxidant folic acid, has been found to significantly reduce serum homocysteine and to lower circulating levels of Hsp72, thus improving the condition of such individuals (Hunter-Lavin et al. 2004a).

### **1.12.5 Neurodegenerative diseases**

Increasing evidence suggests a critical role for HSPs in neurodegenerative diseases, such as AD (Renkawek et al. 1994; Sakahira et al. 2002; Tiraboschi et al. 2004) and HD (Merienne et al. 2003). These diseases appear to involve protein misfolding and an early impairment of the stress response, which in turn compromise the function and survivability of neurons (Scott, 2009). AD is a slow and progressive physical condition affecting the brain, characterised by memory loss and dementia. Its causes are not fully understood, but research indicates that AD is associated with neuritic plaque formation and neurofibrillary tangles in the brain as a result of neuronal loss *via* apoptosis and necrosis induced by  $\beta$ -amyloid protein (Tiraboschi et al. 2004). Multiple studies also implicate

oxidative stress as an important event in the progression of AD (Markesbery, 1997), in particular the increased occurrence of protein modification by ROS compared to control patients (Markesbery, 1997). One proposed mechanism to protect cells from oxidative stress is the expression of HSPs in particular Hsp27 and Hsp70. HSP expression has been studied in the brain of patients with AD and was found significantly increased in the temporal cortex of AD patients compared to control patients (Stege et al. 1999; Yoo et al. 1999) and were found to suppress the toxicity of aberrantly folded proteins (Wilhelmus et al. 2006).

HD is an autosomal-dominant neurodegenerative disease characterised by psychiatric disturbances and dementia (Landles & Bates, 2004). It is caused by the nuclear aggregation of JNK phosphatase M3/6 in neurones, specifically within the striatum and cortex regions (Merienne et al. 2003). The aggregation is a result of heat denaturation or an accumulation in cells of the abnormal polypeptide, mutant huntingtin which contains expanded polyglutamine stretches (Merienne et al. 2003). It has been suggested that Hsp72 binds to the huntingtin fragment, which causes the titration of Hsp72 from a complex with the M3/6 phosphatase, leading to aggregation and inactivation. Merienne and co-workers (2003) found reduced levels of intracellular Hsp72 in cells expressing the mutant huntingtin fragment. However, over-production of Hsp72 protects the M3/6 phosphatase in cells that express the huntingtin fragment with extended polyglutamine, by suppressing JNK activation, through a similar mechanism seen during the stress response (Merienne et al. 2003), suggesting a protective role for elevated levels of intracellular Hsp72.

#### **1.12.6 Cancer**

A wide range of human cancer types have been found to express elevated levels of one or more HSPs (Morimoto et al. 1991). HSPs regulate the function and stability of other proteins involved in the process of apoptosis (Neckers & Ivy, 2003), thereby suggesting HSP expression as a protective mechanism, preventing spontaneous apoptosis associated with malignancy and apoptosis induced by chemotherapy or radiation therapy. This suggests the use of these proteins as potential biomarkers, reflecting the presence of some disease states, disease progression, tumour cell proliferation and differentiation (Ciocca et al. 1993b;

Cornford et al. 2000). HSPs are expressed in malignant cells and tissues where their expression is often associated with poor prognosis and tumour cell proliferation and differentiation (Ciocca et al. 1993b; Kim et al. 1998; Piura et al. 2002). Including: Hsp27 in gastric, liver, and prostate carcinoma and endometrial osteosarcomas (Harrison et al. 1991; Ciocca et al. 1993b; Fuller et al. 1994), Hsp60 in lung cancer and Hodgkin's disease (Wong & Wispe, 1997; Hsu & Hsu, 1998), Hsp72 in breast, endometrial, uterine, cervical and bladder carcinomas (Ciocca et al. 1993a; Ralhan & Karu, 1995; Santarosa et al. 1997), and Hsp90 expression in breast tumours and lung cancer (Jameel et al. 1992; Wong & Wispe, 1997; Neckers & Ivy, 2003) and in patients with leukaemia and Hodgkin's disease (Yufu et al. 1992; Hsu & Hsu, 1998).

HSPs are also expressed in the serum of patients, together with their auto-antibodies. Including: Hsp27 and anti-Hsp27 detected in the serum of some cancer patients (Conroy et al. 1998; Korneeva et al. 2000), such as breast and prostate cancer (Abe et al. 2004), where their expression facilitates immune responses (Fanelli et al. 1998). On the contrary, studies have also implicated increased serum levels of HSP antibodies with improved survival, suggesting extracellular HSPs to also exert activities which suppress anti-cancer immune functions through the innate or adaptive immune response. They have been described to have peptide-carrier function, cytokine-inducing effects, and to stimulate NK cell activity which results in immunogenic cancer cell death (Conroy et al. 1998; Korneeva et al. 2000; Didelot et al. 2007; Schmitt et al. 2007).

Extracellular HSPs are used within many immunotherapy protocols, stemming from their ability to bind in a stable manner to antigenic peptides, which are released from dying tumour cells or are used as components of anti-cancer vaccines. HSPs bind to these peptides of tumour cells during antigen processing by APCs to CD8<sup>+</sup> T cells (Srivastava et al. 2002b). Studies reveal HSPs to act at multiple stages during tumour antigen presentation, in order to enhance the generation of CD8<sup>+</sup> T cell-mediated immunity and lead to regression of primary and metastatic tumours. Two strategies for therapy have been proposed; pharmacological modification of HSP expression or activity; and the use of HSPs in anti-cancer vaccines, exploiting their ability to act as immunological adjuvants (Neckers & Ivy, 2003; Sreedhar & Csermely, 2004b; Bausero et al. 2005).

### 1.13 Aims and Objectives

This thesis aims to investigate the different roles of HSPs in response to various stress stimuli, in terms of inducing apoptotic cell death, cyto-protection, and/or immune regulation, and the mechanism(s) involved, using cultured and human peripheral blood cells.

The objectives of this thesis are:

- To determine the effects of an elevation of temperature in cultured U937 cells *in vitro* and human peripheral blood cells *ex vivo*, and to examine under the same conditions the intracellular and extracellular localisation of HSPs with the intention of determining their physiological roles.
- To determine whether extracellular bovine Hsp70 or recombinant human Hsp72 can bind to cell surface receptors and be taken up by cultured U937 and human peripheral blood cells under control conditions.
- To determine the contributions of extracellular Hsp70 or Hsp72 in cyto-protection from environmental, and physiological stressors such as heat shock, osmotic stress and uric acid exposure.
- To determine the cell surface receptors for extracellular Hsp70 or Hsp72.
- To determine whether extracellular human Hsp72 or cell derived Hsp72 are capable of interacting with, and activating U937 macrophages and/or human leukocytes, and whether they can stimulate an immune response.

## Chapter 2

### Materials and Methods

#### 2.1 Consumables and Equipment

##### **0.6 mL Microcentrifuge Tubes**

Thermo Fisher Scientific Inc.

Product Number. TUL-918-010X

##### **1.5 mL Microcentrifuge Tubes**

Thermo Fisher Scientific Inc.

Product Number. TUL-918-014G

##### **12-well Cell Culture Plates**

Thermo Fisher Scientific Inc.

Product Number. TKT-520-070H

##### **15 mL Centrifuge Tubes**

StarLab Ltd.

Product Number. E1415-0200

##### **24-well Cell Culture Plates**

Thermo Fisher Scientific Inc.

Product Number. TKT-520-100B

##### **25 cm<sup>2</sup> Cell Culture Flasks**

Thermo Fisher Scientific Inc.

Product Number. 136196

##### **48-well Cell Culture Plates**

Thermo Fisher Scientific Inc.

Product Number. TKT-522-070S

##### **50 mL Centrifuge Tubes**

StarLab Ltd.

Product Number. E1450-0200

##### **96-well Cell Culture Plates**

Thermo Fisher Scientific Inc.

Product Number. DPS-130-010N

**96-well TC-treated Black Clear Bottomed Plates**

Thermo Fisher Scientific Inc.

Product Number. DPS 130-020K

**384-well Polypropylene Black Assay Plates**

Corning.

Product Number. 07-200-765

**BD Eclipse Blood Collection Needle, 21g**

Southern Syringe Services Ltd.

Product Number. 368609

**BD Vacutainer® One Use Holder**

Southern Syringe Services Ltd.

Product Number. 364815

**BD Vacutainer® Whole Blood Tube, K<sub>2</sub>EDTA, 6 mL**

Southern Syringe Services Ltd.

Product Number. 367873

**Bio-Rad ChemiDoc XRS Molecular Imaging System**

Bio-Rad Laboratories Ltd.

Product Number. 170-8070

**Bio-Tek Synergy™ HT Multi-Detection Microplate Reader**

Labtech International Ltd.

Product Number. SIAFR

**Bright-Line™ Haemocytometer**

Sigma-Aldrich Ltd.

Product Number. Z359629

**Circulating Water Bath**

Wolf Laboratories Ltd.

Product Number. GD100-P5

**Coverslips**

Thermo Fisher Scientific Inc.

Product Number. 22-037-169

**Cryovials®**

Thistle Scientific

Product Number. T301-1

**Dynex Ultrawash PLUS™ Plate Washer**

Jencons (Scientific) Ltd.

Product Number. 701-005

**E100 Binocular Microscope**

Jencons (Scientific) Ltd.

Product Number. 450-951

**Extra Thick Blot Paper**

Bio-Rad Laboratories Inc.

Product Number. 170-3965

**FACS Canto™ Dual Laser Flow Cytometer**

BD Biosciences™.

Product Number. 337175

**Glass Slides**

Thermo Fisher Scientific Inc.

Product Number. 12-550-15

**Heparin Beads**

ADAR Biotech.

Product Number. 6024-5/10/25

**Hermle Z323K Refrigerated Centrifuge**

VWR International Ltd.

Product Number. 521-0221

Including:

**Swing-out Rotor (8 X 15 mL)**

Product Number. 521-0189

**Fixed-angle Rotor (24 X 1.5 mL)**

Product Number. 521-0201

**Fixed-angle Rotor (8 X 50 mL)**

Product Number. 521-0194

**High Speed Mini Orbital Shaker**

Wolf Laboratories Ltd.

Product Number. SSM5

**HTS Transwell-96 Well Permeable Support System (3 µM)**

Thermo Fisher Scientific Inc.

Product Number. HTS-106-010V

**HTS Transwell-96 Well Permeable Support System (8 µM)**

Thermo Fisher Scientific Inc.

Product Number. HTS-106-050J



**Inverted TE2000-U Microscope System**

Nikon Corporation Ltd:

**Eclipse TE2000-U Basic Unit**

Product Number. MEA51010

**Eposcopic Fluorescence Attachment (Hg)**

Product Number. MEE54000

**CFI Plan Fluor ELWD Objectives.**

Product Numbers. MRH38220/MRH38420/MRH18620

**Hamamatsu Orca-285 Digital CCD Camera**

Product Number. 1HMOC285

**Coolpix Digital Colour Camera**

Product Number. 85400RUK

**IPLAB Suite Software**

Product Number. 1SCSUITE

**LMS Series 1 Cooled Incubator**

Wolf Laboratories Ltd.

Product Number. 305

**Microplate 96-Well V Bottom Polystyrene Clear**

Thermo Fisher Scientific Inc.

Product Number. FB-56424

**Microplate 384-Well Square Shape Polystyrene Solid Black**

Thermo Fisher Scientific Inc.

Product Number. FB-58050

**Mini Trans-Blot Electrophoretic Transfer Cell**

Bio-Rad Laboratories Inc.

Product Number. 170-3930

**Mini-PROTEAN™ II Electrophoresis Cell**

Bio-Rad Laboratories Inc.

Product Number. 165-2940.

**Nitrocellulose Membrane, 0.45µm**

Bio-Rad Laboratories Inc.

Product Number. 162-0115

**Non-binding 96-well Plates**

Thermo Fisher Scientific Inc.

Product Number. FB56426

**Nunc Immuno Maxisorp 96-well Plates**

Thermo Fisher Scientific Inc.

Product Number. DIS-917-030J

**Pasteur Pipettes**

Thermo Fisher Scientific Inc.

Product Number. FB55348

**PowerPac™ 3000 Power Supply**

Bio-Rad Laboratories Inc.

Product Number. 165-5057

**Sigma 1-14 Microcentrifuge**

Wolf Laboratories Ltd.

Product Number. 10016

**Temperature-controlled, Stirred Water Bath, GD100-S5**

Thermo Fisher Scientific Inc.

Product Number. BLE-650-010G

**Thermal Gradient Bar**

In house design.

**Ultrafree®-CL Microcentrifuge Filters NMWL 10,000 kDa**

Sigma-Aldrich Ltd.

Product Number. M1536

**Ultrafree®-MC Microcentrifuge Filters NMWL 5,000 kDa**

Sigma-Aldrich Ltd.

Product Number. M0286

**Vortex Mixer, mini**

Thermo Fisher Scientific Inc.

Product Number. GBI-900-010E

**Whatman #1 Filter Paper**

Thermo Fisher Scientific Inc

Product Number. FB59027

## 2.2 Chemicals and Reagents

### **Acrylamide**

VWR International Ltd.

Product Number. 442994J

### **Adenosine 5'-Triphosphate Disodium Salt**

Sigma-Aldrich Ltd.

Product Number. A6559

### **$\epsilon$ -Amino-n-caproic Acid**

Sigma-Aldrich Ltd.

Product Number. A7824

### **Ammonium Chloride**

Sigma-Aldrich Ltd.

Product Number. A9434

### **Ammonium Hydroxide Solution**

Sigma-Aldrich Ltd.

Product Number. 338818

### **Ammonium Persulphate**

Sigma-Aldrich Ltd.

Product Number. A3678

### **Annexin V: FITC Conjugated Apoptosis Detection Kit I**

BD Biosciences™.

Product Number. 556420

### **Anti-Goat IgG (whole molecule)-FITC Antibody Produced in Rabbit**

Sigma-Aldrich Ltd.

Product Number. F2016

### **Anti-Goat IgG (whole molecule)-R-PE Antibody Produced in Rabbit**

Sigma-Aldrich Ltd.

Product Number. P7878

### **Anti-Goat IgG (whole molecule) Peroxidase-Produced in Rabbit**

Sigma-Aldrich Ltd.

Product Number. A5420

### **Anti-Goat IgG-FITC Isotype Control**

abcam®.

Product Number. 37374

**Anti-Goat IgG-R-PE Isotype Control**

abcam®.

Product Number. 37377

**Anti-Mouse IgG (whole molecule)-FITC Antibody Produced in Goat**

Sigma-Aldrich Ltd.

Product Number. F0257

**Anti-Mouse IgG (whole molecule)-R-PE Antibody Produced in Goat**

Sigma-Aldrich Ltd.

Product Number. P9670

**Anti-Mouse IgG1-FITC Isotype Control**

DAKO.

Product Number X0927

**Anti-Mouse IgG1-R-PE Isotype Control**

DAKO.

Product Number X0928

**Anti-Mouse IgG Peroxidase-Produced in Goat**

Sigma-Aldrich Ltd.

Product Number. A5278

**Anti-Rabbit IgG-FITC Isotype Control**

eBioscience Inc.

Product Number 11-4614

**APC Mouse Anti-Human CD15**

BD Biosciences™.

Product Number. 551376

**Benzamidine**

Sigma-Aldrich Ltd.

Product Number. B6506

**BD FACS Lysing Solution**

BD Biosciences™.

Product Number. 349202

**Bio-Rad Protein Assay Dye Reagent Concentrate**

Bio-Rad Laboratories Inc.

Product Number. 500-0006

**Bovine Serum Albumin (BSA)**

Sigma-Aldrich Ltd.

Product Number. A7906

**Brij®-98**

Sigma-Aldrich Ltd.

Product Number. A436240

**Bromophenol Blue**

Thermo Fisher Scientific Inc.

Product Number. B/4630/44

**Calcium Chloride Dihydrate**

Sigma-Aldrich Ltd.

Product Number. C3306

**Camptothecin**

Sigma-Aldrich Ltd.

Product Number. C9911

**CellTiter® Aqueous One Solution Cell Proliferation Assay**

Promega UK Ltd.

Product Number. G3580

**CD14 Monoclonal Antibody Produced in Mouse**

Sigma-Aldrich Ltd.

Product Number. C7673

**CD36 (N15) Polyclonal Antibody Produced in Goat**

Santa Cruz Biotechnology.

Product Number. sc-5522

**CD77 Monoclonal Antibody Produced in Mouse**

BD Biosciences™.

Product Number. 551352

**Cytofix/Cytoperm Fixation and Permeabilization Solution**

BD Biosciences™.

Product Number. 554722

**4-Diazabicyclo[2.2.2]octane (DABCO)**

Sigma-Aldrich Ltd.

Product Number. D2522

**1, 3-Dioxane 97 %**

Sigma-Aldrich Ltd.

Product Number. D283061

**D-Glucose 6-Phosphate Dipotassium Salt Hydrate**

Sigma-Aldrich Ltd.

Product Number. G7375

**Dimethyl Sulfoxide (DMSO)**

Sigma-Aldrich Ltd.

Product Number. D2650

**DL-Dithiothreitol**

Sigma-Aldrich Ltd.

Product Number. D0632

**Dulbecco's Phosphate Buffered Saline (DPBS)**

Lonza.

Product Number. BE17513F

**Ethylenediaminetetraacetic Acid, Anhydrous (EDTA)**

Sigma-Aldrich Ltd.

Product Number. E6758

**Enzolyte™ Rh110 Caspase-3 Assay Kit**

Anaspec Inc.

Product Number. 71141

**Ethanol**

Sigma-Aldrich Ltd.

Product Number. E459836

**ExtrAvidin® Peroxidase Conjugate**

Sigma-Aldrich Ltd.

Product Number. E2886

**ExtrAvidin®-FITC Conjugate**

Sigma-Aldrich Ltd.

Product Number. E2886

**FITC Rabbit Anti-Active Caspase-3**

BD Biosciences™.

Product Number. 559341

**FITC Mouse Anti-Human CD36**

BD Biosciences™.

Product Number. 555454

**FITC Mouse Anti-Human CD77**

BD Biosciences™.

Product Number. 551353

**FITC Mouse Anti-Human CD91**

BD Biosciences™.

Product Number. 550496

**Foetal Bovine Serum (FBS)**

Lonza.

Product Number. 14-810F

**Formaldehyde Solution 37 wt. % in H<sub>2</sub>O**

Sigma-Aldrich Ltd.

Product Number. 533998

**Fucoidan from *Fucus vesiculosus* Crude**

Sigma-Aldrich Ltd.

Product Number. F5631

**Glucose-6-phosphate Dehydrogenase from baker's yeast (*S. cerevisiae*)**

Sigma-Aldrich Ltd.

Product Number. G6378

**Glycerol**

Sigma-Aldrich Ltd.

Product Number. G5516

**Glycine**

VWR International Ltd.

Product Number. 101196X

**Heat Shock Protein 70 from bovine brain (Hsp70)**

Sigma-Aldrich Ltd.

Product Number. H9776

**HEPES**

Sigma-Aldrich Ltd.

Product Number. H3375

**Histopaque®-1077 Hybri-max Solution**

Sigma-Aldrich Ltd.

Product Number. H8889

**Hsp27 Mouse Monoclonal Antibody: PE Conjugate**

Cambridge Bioscience.

Product Number. SPA-800PE

**Hsp27 Mouse Monoclonal Antibody (G3.1): Biotin**

Assay Designs Inc.

Product Number. SPA-800B

**Hsp60 Mouse Monoclonal Antibody (LK2): PE Conjugate**

Cambridge Bioscience.

Product Number. SPA-807PE

**Hsp60 Rabbit Polyclonal Antibody**

Assay Designs Inc.

Product Number. SPA-805

**Hsp70 (Hsp72) Mouse Monoclonal Antibody: FITC conjugated**

Cambridge Bioscience.

Product Number. SPA- 810FITC

**Hsp70 (Hsp72) Recombinant Human Protein**

Assay Designs Inc.

Product Number. NSP-555

**Hsp70 Monoclonal Antibody against TKD Peptide (cm.Hsp70.1)**

Purchased from Multi-immune Labs, Munich, Germany.

**Hsp72 Mouse Monoclonal Antibody raised against bovine Hsp70 (Sigma, H9776)**

A kind gift from Dr Torsten Nygård, Faculty of Agricultural Sciences, Aarhus University, Denmark.

**Hsp72 (Hsp72-DEG-E1) affinity purified Sheep Polyclonal Antibody against a sequence from HSPA1A**

Purified in House.



**Hsp90 Mouse Monoclonal Antibody: PE Conjugate**

Cambridge Bioscience.

Product Number. SPA-830PE

**Hsp90 Mouse Monoclonal Antibody**

Assay Designs Inc.

Product Number. SPA-830

**Human IL-10 (Interleukin-10) ELISA Ready-SET-Go! Kit**

eBioscience Inc.

Product Number. 88-7106

**Human TNF- $\alpha$  (Tumour Necrosis Factor alpha) ELISA Ready-SET Go! Kit**

eBioscience Inc.

Product Number. 88-7346

**Hydrochloric Acid, 37 %**

Sigma-Aldrich Ltd.

Product Number. H1758

**Imperial Protein Stain**

Pierce Biotechnology Inc.

Product Number. 24615

**L-Glutamine Solution**

Lonza.

Product Number. 17-605E

**LOX-1 Mouse Monoclonal Antibody**

abcam®.

Product Number. ab-81709

**Lipopolysaccharide, from *Escherichia coli* Serotype 026:B6**

Sigma-Aldrich Ltd.

Product Number. L8274

**Macrophage Scavenger Receptor I Goat Polyclonal Antibody**

abcam®.

Product Number. ab-6417

**Maleic Anhydride**

Sigma-Aldrich Ltd.

Product Number. M0625

**Mem-PER Eukaryotic Membrane Protein Extraction Kit**

Thermo Fisher Scientific Inc.

Product Number. 89826

 **$\beta$ -Mercaptoethanol**

Thermo Fisher Scientific Inc.

Product Number. M/P200/05

**Methanol**

VWR International Ltd.

Product Number. 152506X

**N-Formyl-L-methionyl-L-leucyl-L-phenylalanine: N-formyl-MLF (FMLP)**

Sigma-Aldrich Ltd.

Product Number. F3506

 **$\alpha$ -Nicotinamide Adenine Dinucleotide Phosphate Sodium Salt**

Sigma-Aldrich Ltd.

Product Number. N2010

**N,N,N',N'-Tetramethylethylenediamine (TEMED)**

Sigma-Aldrich Ltd.

Product Number. T9281

**N'N'-Methylenebisacrylamide**

VWR International Ltd.

Product Number. 4433003N

**Orthophosphoric Acid**

In house.

**Paraformaldehyde Powder 95 %**

Sigma-Aldrich Ltd.

Product Number. 158127

**PE Mouse Anti-Human CD3**

BD Biosciences™.

Product Number. 555340

**PE-Cy™7 Mouse Anti-Human CD14**

BD Biosciences™.

Product Number. 557742

**Phenylmethylsulfonyl Fluoride**

Sigma-Aldrich Ltd.

Product Number. P7626

**Phorbol 12-Myristate 13-Acetate (PMA)**

Sigma-Aldrich Ltd.

Product Number. P1585

**Phosphate Buffered Saline without  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$** 

Lonza.

Product Number. BE17-516F

**Polyinosinic Acid (Poly (I)) Potassium Salt**

Sigma-Aldrich Ltd.

Product Number. P4154

**Potassium Chloride**

Sigma-Aldrich Ltd.

Product Number. P5405

**Potassium Dihydrogen Orthophosphate**

VWR International Ltd.

Product Number. 102034B

**Precision Plus<sup>®</sup> Protein Dual Colour Standards**

Bio-Rad Laboratories Inc.

Product Number. 161-0374

**Precision Plus<sup>®</sup> Protein Unstained Standards**

Bio-Rad Laboratories Inc.

Product Number. 161-0363

**ProLong<sup>®</sup> Gold with antifade reagent with DAPI**Invitrogen<sup>™</sup>.

Product Number. P-36935

**Propidium Iodide (PI) Minimum 95 %**

Sigma-Aldrich Ltd.

Product Number. P4170

**Protease Inhibitor Cocktail 100X**

Sigma-Aldrich Ltd.

Product Number. P8340

**RPMI-1640 Medium**

Lonza.

Product Number. BE12-702F

**RPMI-1640 Medium, Phenol Red-Free**

Lonza.

Product Number. BE12-918F

**Sodium Bicarbonate**

Sigma-Aldrich Ltd.

Product Number. S6297

**Sodium Carbonate**

Sigma-Aldrich Ltd.

Product Number. S7795

**Sodium Chloride**

Sigma-Aldrich Ltd.

Product Number. S7653

**Sodium Deoxycholate**

Sigma-Aldrich Ltd.

Product Number. D6750

**Sodium Dihydrogen Orthophosphate 1-hydrate**

VWR International Ltd.

Product Number. 102454R

**Sodium Dodecyl Sulphate**

VWR International Ltd.

Product Number. 442444H

**Sodium Hydroxide**

Sigma-Aldrich Ltd.

Product Number. S8045

**Sodium Phosphate Buffer**

Sigma-Aldrich Ltd.

Product Number. 342483

**Sodium Pyrophosphate Buffer**

Sigma-Aldrich Ltd.

Product Number. 221368

**Sucrose**

Sigma-Aldrich Ltd.

Product Number. S7903

**SuperSignal® West Pico Chemiluminescent Substrate**

Pierce Biotechnology Inc.

Product Number. 30477

**SuperSignal® West Femto Trial kit**

Pierce Biotechnology Inc.

Product Number. 34094

**3,3',5,5'- Tetramethylbenzidine (TMB) Substrate**

Cheshire Sciences (UK) Ltd.

Product Number. UP664781

**Thimerosal**

Sigma-Aldrich Ltd.

Product Number. T8784

**Tris (hydroxymethyl) Methylamine**

VWR International Ltd.

Product Number. 443866G

**Triton® X-100**

Sigma-Aldrich Ltd.

Product Number. T8787

**Trypan Blue Solution (0.4 %)**

Sigma-Aldrich Ltd.

Product Number. T1854

**Tween® 20**

Sigma-Aldrich Ltd.

Product Number. P1379

**Uric Acid**

Sigma-Aldrich Ltd.

Product Number. U0881

**U937 Human Cell Line**

European Collection of Cell Cultures.

Product Number. 85011440

### **2.2.1 Cell Extraction Buffer**

0.315 g (w/v) of tris base, 0.004 g of ethylenediaminetetraacetic acid (EDTA) and 0.01 g of DL-dithiothreitol were dissolved and made up to 100 mL with distilled water (dH<sub>2</sub>O) and the pH was adjusted to 7.4 with 1 M hydrochloric acid (HCl). This solution was then supplemented with 0.035 g of phenylmethylsulfonyl fluoride (PMSF), 0.065 g of  $\epsilon$ -amino-n-caproic acid, 0.016 g of benzamidine and 0.1 % (v/v) Triton X-100, supplemented with 1 mL of 100X protease inhibitor cocktail. This solution was prepared up to one month in advance and stored at 4°C.

### **2.2.2 Flow Cytometry Solutions**

#### **Antibody Wash Buffer (v/v)**

5 % foetal bovine serum (FBS) was added to Dulbecco's phosphate buffered saline (DPBS), pH 7.2.

#### **Binding Buffer 10X (w/v)**

0.1 M HEPES, 1.4 M sodium chloride and 25 mM of calcium chloride dehydrate were added to 1 L of dH<sub>2</sub>O and the pH was adjusted to 7.4 with 1 M HCl. A 1X working dilution was obtained by diluting 1:10 with DPBS, pH 7.2.

#### **Erythrocyte Lysing Buffer 10X (w/v) Solution**

8.02 g of ammonium chloride, 0.84 g of sodium carbonate and 0.37 g of EDTA disodium were dissolved in 50 mL of dH<sub>2</sub>O. A 1X working dilution was obtained by diluting 1:10 with dH<sub>2</sub>O.

#### **Paraformaldehyde 4 % (w/v)**

2 g of paraformaldehyde and 100  $\mu$ L of 5 M sodium hydroxide (NaOH) were added to 40 mL of DPBS. This was incubated at 56°C in a heated water bath until the paraformaldehyde was completely dissolved. The pH was adjusted to 7.4 with 100  $\mu$ L 5 M HCl and the solution was made up to 50 mL with DPBS. This was stored at 4°C for a maximum of seven days.

### **2.2.3 DABCO Mounting Media**

1 % (w/v) 4-Diazabicyclo[2.2.2]octane (DABCO) was added to 90/10 % (v/v) of glycerol and PBS.

### **2.2.4. Phosphate Buffered Saline (PBS), pH 7.2 (w/v)**

8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.24 g of potassium dihydrogen orthophosphate and 1.44 g of sodium dihydrogen orthophosphate were added to 1 L of dH<sub>2</sub>O and the pH adjusted to 7.2 with 1 M HCl.

### **2.2.5 Propidium Iodide (PI) Stock Solution (1 mg/mL) (w/v)**

0.001 g of PI was dissolved in 1 mL of PBS, pH 7.4. 100 µg/mL stock solution aliquots were prepared and stored at -20°C for single use.

### **2.2.6 MTS Working Solution (w/v)**

0.042 g of MTS was dissolved in 21 mL of DPBS and the pH was adjusted to 6.5 with 1 M HCl. PES stock solution was prepared by dissolving 0.0092 g of PES in 10 mL of DPBS. The MTS working solution was achieved by adding 1 mL of PES stock solution to 20 mL of MTS stock solution and was stored at -20°C.

### **2.2.7 SDS-PAGE Buffers**

#### **1.5 M Tris-HCl, pH 8.8 (w/v)**

18.50 g of tris base was added to 40 mL of dH<sub>2</sub>O and the pH was adjusted to 8.8 with 1 M HCl. This solution was then made up to 100 mL with dH<sub>2</sub>O.

#### **0.5 M Tris-HCl, pH 6.8 (w/v)**

3.00 g of tris base was added to 25 mL of dH<sub>2</sub>O and the pH adjusted to 6.8 with 1 M HCl. This solution was then made up to 50 mL with dH<sub>2</sub>O.

#### **Acrylamide-bis (w/v)**

30.00 g of acrylamide and 0.80 g of N'N' bismethylene acrylamide was added to 100 mL of dH<sub>2</sub>O; this was stirred thoroughly and then filtered through Whatman #1 filter paper. The solution was stored at 4°C in the dark for a maximum of thirty days.

**10 % (w/v) Sodium Dodecyl Sulphate (SDS) Solution**

10.0 g of SDS was dissolved in 100 mL of dH<sub>2</sub>O with gentle stirring.

**10 % (w/v) Ammonium Persulphate**

0.1 g of ammonium persulphate was added to 1 mL of dH<sub>2</sub>O. This was freshly prepared when required.

**0.05 % (w/v) Bromophenol Blue**

0.01 g of bromophenol blue was added to 20 mL of dH<sub>2</sub>O.

**Non-reducing Sample Buffer (w/v)**

2.40 g of sucrose, 2.0 mL of 0.5 M Tris-HCl pH 6.8, 2.0 mL of 10 % SDS solution, and 0.40 mL of 0.05 % bromophenol blue were added to 9.10 mL of dH<sub>2</sub>O and mixed thoroughly.

**Reducing Sample Buffer (w/v)**

Non-reducing sample buffer with the addition of 0.02 g of DL-dithiothreitol.

**Electrode Buffer, pH 8.3 (w/v)**

5.4 g of tris base, 25.92 g of glycine and 9 mL of 10 % SDS solution was added to 900 mL of dH<sub>2</sub>O, stirred thoroughly and the pH was adjusted to 8.3 with 1 M HCl. This was prepared a day prior to use and stored at 4°C.

**2.2.8 Western Blotting Buffers****Transfer Buffer (w/v)**

3.03 g of tris base, 14.4 g of glycine and 200 mL of methanol was made up to 1 L with dH<sub>2</sub>O. This was prepared a day prior to use and stored at 4°C.

**Tris Buffered Saline (TBS) (w/v)**

2.42 g of tris base and 29.22 g of NaCl was added to 750 mL of dH<sub>2</sub>O. The pH was adjusted to 7.5 then made up to 1 L with dH<sub>2</sub>O.



**Washing Solution (TTBS) (w/v)**

0.05 % (v/v) of Tween<sup>®</sup> 20 was added to 500 mL of TBS and stirred thoroughly.

**Blocking Solution (w/v)**

1 % bovine serum albumin (BSA) was added to 50 mL of TBS and stirred thoroughly.

**Antibody Buffer (w/v)**

1 % BSA was added to 100 mL of TTBS and stirred thoroughly.

**2.2.9 ELISA Buffers****0.1 M (w/v) Sodium bicarbonate buffer, pH 9.6**

10.6 g of sodium carbonate and 8.4 g of sodium bicarbonate were added to 1 L of dH<sub>2</sub>O and the pH was adjusted to 9.6 with 1 M HCL.

**Blocking Buffer (w/v)**

0.5 % BSA was dissolved in PBS (Section 2.2.4).

**Wash Buffer**

0.05 % (v/v) Tween<sup>®</sup>20 and 0.01 % (w/v) thimerosal was added to PBS, pH 7.2.

**Detector Antibody**

The detector antibody was kindly donated by Dr Tortsen Nygård, from the Faculty of Agricultural Sciences, Aarhus University, Denmark. The antibody (mouse monoclonal) was raised against bovine Hsp70 (H9776, Sigma-Aldrich Ltd) at a concentration of 1 mg/mL in PBS with 0.01 % (w/v) thimerosal.

**1 M (v/v) Phosphoric Acid**

6.8 mL of orthophosphoric acid was diluted with dH<sub>2</sub>O up to 100 mL.

## **2.3 Methods**

### **2.3.1 Cell culture conditions.**

All cell culture and experimental treatments were performed using aseptic technique in a class II tissue culture hood. All cells were grown or incubated at 37°C in a humidified atmosphere with 5 % CO<sub>2</sub> unless otherwise stated.

### **2.3.2 Preparation of cell culture media (10 % RPMI).**

RPMI-1640 medium was equilibrated at 37°C and then supplemented with 10 % (v/v) FBS. 2 mL aliquots of 10 % RPMI were regularly transferred to 12-well tissue culture plates and examined under an inverted light microscope for infection and integrity of the cell culture media.

### **2.3.3 Thawing of frozen cell lines.**

1 mL vials of cells were removed from liquid nitrogen and rapidly thawed at 37°C until almost completely defrosted. 9 mL of pre-warmed 10 % RPMI was then dispensed into a 25 cm<sup>2</sup> tissue culture flask and the thawed cell suspension was added. The flask was observed under an inverted light microscope for viability and incubated at 37°C with 5 % CO<sub>2</sub>.

### **2.3.4 Growth of U937 cell line.**

Human monocytic U937 cells were cultured in 25 cm<sup>2</sup> plastic tissue culture vented flasks using 10 % RPMI and seeded at a density of  $2-9 \times 10^5$  cells/mL. Cells were routinely passaged every 3-4 days or when confluent, and observed for viability using trypan blue exclusion (Section 2.3.7).

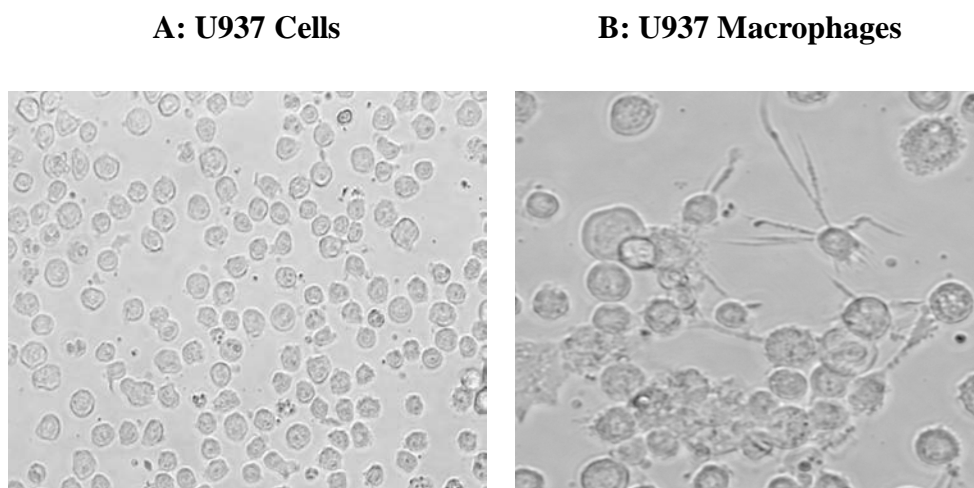
### **2.3.5 Preparation of heat-inactivated serum (HI-FBS).**

FBS was heat-inactivated to inactivate heat sensitive components such as growth factors and complement proteins which may adversely affect experimental results. FBS was allowed to thaw at 37°C before being placed in a 56°C water bath for 30 minutes then stored at -20°C until required.

### **2.3.6 Transformation of U937 cells into U937 macrophages.**

Human monocytic U937 cells were grown as described previously (Section 2.3.4) and prior to experimental treatments were removed from cell culture flasks and viability was tested as described in section 2.3.7. Transformation of U937 cells into U937 macrophages was achieved using a suspension of cells actively growing in the log phase and at >95 % viability. Cells were centrifuged at 500 *g* for 3 minutes at 25°C, washed once with 10 % RPMI and re-suspended in phenol red-free RPMI supplemented with 10 % (v/v) heat-inactivated FBS (10 % HI-RPMI), containing 10 ng/mL phorbol 12-myristate 13-acetate (PMA). Cells were plated out into 12-well cell culture plates at  $5 \times 10^5$  cells/mL (1 mL/well) and incubated for 48 hours at 37°C in a humidified atmosphere with 5 % CO<sub>2</sub> to enable differentiation of cells. After 48 hours, the media was removed and cells were gently rinsed twice with 10 % HI-RPMI, then 1 mL/well of fresh 10 % HI-RPMI was added. U937 macrophages were then ready for experimental treatments.

When transformed with PMA, U937 cells became adherent to well surfaces, sometimes forming in clusters, and they exhibited morphological changes that included becoming larger in appearance, granular with large lysosomes whilst some cells presented protrusions of the cytoplasm known as pseudopodia and the majority of cells ceased to proliferate. In order to detach the U937 macrophages from the surface of the cell culture plate, the medium was carefully pipetted up and down, leaving the cells in suspension. All observations were consistent with maturation of monocytes to macrophages (Figure 2.1).



**Figure 2.1: (A) normal U937 cells and (B) PMA transformed U937 Cells.** U937 cells were transformed into U937 macrophages using 10 ng/mL PMA and incubated for 48 hours at 37°C in a humidified atmosphere with 5 % CO<sub>2</sub>. Brightfield images were generated on a conventional microscope, Eclipse TE2000-U microscope and were acquired at a magnification of x 40.

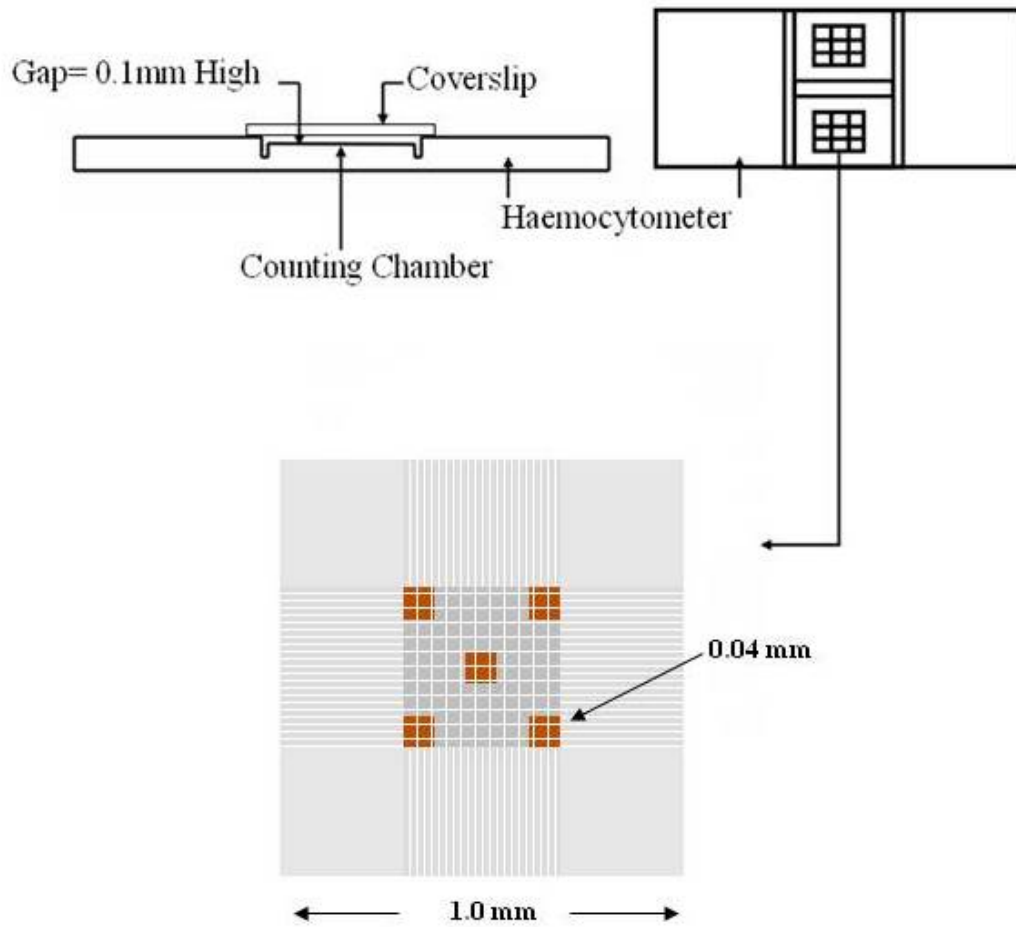
### 2.3.7 Cell counting and viability assay.

Trypan blue is a non-permeable cell membrane DNA dye that is used to determine the numbers of viable cells present in a cell suspension. It is based on the principle that viable cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas necrotic cells allow the dye to pass the cell membrane because of the loss of membrane integrity and stain the DNA inside the cells.

Routinely, 500 µL of cell suspension was diluted 1:1 with trypan blue in a sterile 0.6 mL microcentrifuge tube and incubated for 10 minutes. Cell counts and viability were carried out in duplicate with 50 µL of cell/trypan blue suspension using a haemocytometer and viewed under a light microscope. Cells were counted in the 0.04 mm centre square and in four 0.04 mm corner squares (Figure 2.2), this was repeated twice and an average was taken. The total number of cells per 1 mL was then calculated using the following formula:

$$\text{Total number of cells per mL} = \text{cell count} \times 5 \times \text{dilution factor} \times 10,000$$

A separate count of viable and non-viable cells was also performed and samples which contained >5 % non-viable cells were discarded.



**Figure 2.2: Illustration of a haemocytometer.** (Adapted from Rougem, 2002).

### 2.3.8 Freezing and storage of cell lines.

Cells were routinely frozen down for long term storage to maintain minimal passaging of cells. Sub-confluent cells ( $2-9 \times 10^5$  cells/mL) were transferred to a 15 mL centrifuge tube and centrifuged at 300 *g* for 3 minutes at 25°C to minimise damage to cells. The medium was removed and the cell pellet was re-suspended in 1 mL of ice-cold freeze medium (FBS supplemented with 10 % (v/v) sterile dimethyl sulfoxide (DMSO)). The cell suspension was then transferred to a cryovial and incubated in vapour phase liquid nitrogen for at least 2 hours and then transferred to liquid nitrogen and stored until required.

**2.3.9 Whole blood collection.**

The blood provided for these studies came from healthy volunteers aged 25-50 years. The departmental ethics committee approved this section of study and consent forms were completed by each volunteer. Venous whole blood was collected by venepuncture in 6 mL K<sub>2</sub>EDTA vacutainers and all manipulations were carried out within 2 hours of collection.

**2.3.10 Isolation of leukocytes from whole blood.**

Whole blood was washed with two volumes of DPBS and centrifuged at 500 *g* for 5 minutes at 25°C. The supernatant was discarded and erythrocytes were eliminated using 1X lysing buffer. The lysed whole blood was centrifuged at 500 *g* for 5 minutes at 25°C and the supernatant discarded. The white blood cell pellet was then washed twice with DPBS and the cells were re-suspended with 1 mL of antibody wash buffer (DPBS and 5 % FBS). Leukocytes were then counted using trypan blue exclusion method on a haemocytometer (Section 2.3.7). Experiments were performed when the percentage of dead cells was <1 %. The concentration of viable leukocytes was adjusted to 3 x 10<sup>5</sup> cells/well and the cells were centrifuged at 500 *g* for 5 minutes at 25°C. Supernatant was removed and discarded and the leukocyte pellet was kept on ice for further treatments (Section 2.3.13 and 2.3.14) or for flow cytometric analysis (Section 2.3.20).

**2.3.11 Isolation of erythrocytes from whole blood.**

3 mL aliquots of whole blood were mixed by inversion with 3 mL of DPBS in 15 mL centrifuge tubes and subjected to density centrifugation using Histopaque® 1077 (3 mL, which was carefully layered using a glass pipette underneath the blood/DPBS layer) at 400 *g* for 30 minutes at 25°C. After centrifugation, erythrocytes were isolated by gentle removal of the upper layer of platelet-rich plasma and the peripheral blood mononuclear cell (PBMC) layer with a pasteur pipette to within 0.5 cm of the erythrocyte interface. Erythrocytes were carefully transferred to a new 15 mL sterile centrifuge tube with 1 mL of DPBS and mixed by inversion. This was centrifuged at 350 *g* for 10 minutes at 25°C and the supernatant was discarded. This was repeated twice. The erythrocyte pellet was then re-suspended with 1 mL DPBS and cells counted

using trypan blue exclusion (Section 2.3.7). The cell suspension was then diluted to adjust the cell density to  $1 \times 10^6$  cells/mL in DPBS. The erythrocyte pellet was kept on ice for further treatments (Section 2.3.13 and 2.3.14) or for flow cytometric analysis (Section 2.3.20).

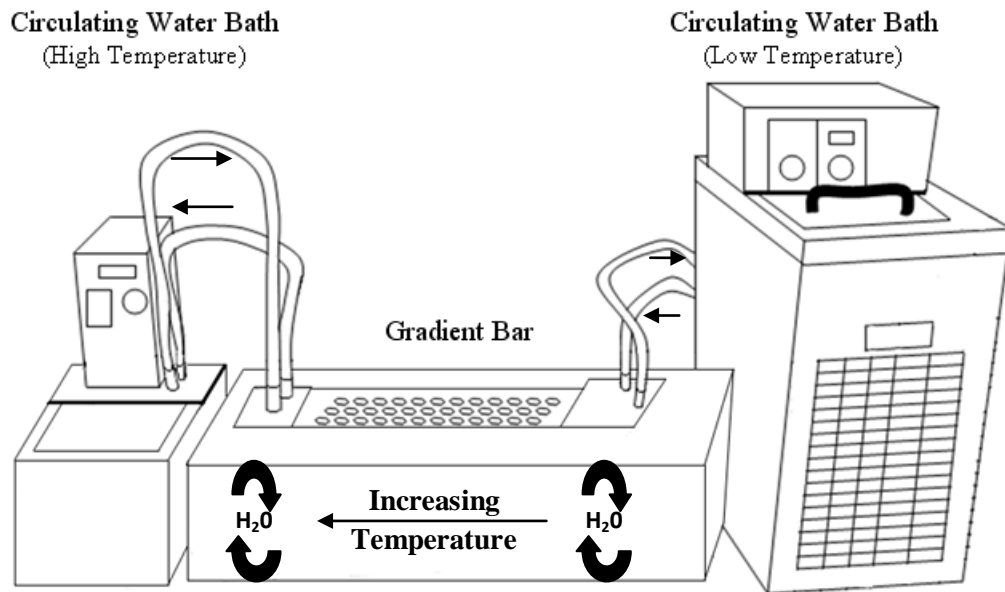
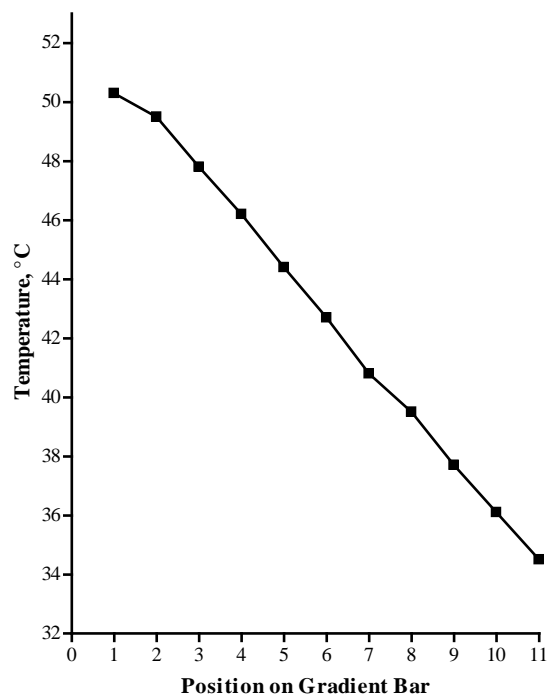
### **2.3.12 Preparation of cells prior to experimental treatments.**

Before treatments, U937 cells were transferred from 25 cm<sup>2</sup> tissue culture flasks into 15 mL sterile centrifuge tubes and centrifuged at 400 g for 5 minutes at 25°C. The supernatant was discarded and the cell pellet was washed once with 10 % RPMI then re-suspended in 10 mL phenol red-free 10 % RPMI. U937 cells were then counted by trypan blue exclusion (Section 2.3.7) and adjusted to a concentration of  $3 \times 10^5$ /mL 24 hours prior to experiments to ensure log-phase growth.

### **2.3.13 *In vitro* heat treatments.**

Two types of heat shock regimes were used for all cells. Cells were transferred from cell culture plates (U937 cells) or 15 mL centrifuge tubes (human leukocytes and erythrocytes) into sterile 1.5 µL microcentrifuge tubes or into sterile 15 mL centrifuge tubes and were exposed to elevated heat treatment using:

- 1) A thermal gradient bar set at the appropriate temperatures for 0-6 hours then transferred to a 37°C incubator with 5 % CO<sub>2</sub> for 1 hour recovery (Figure 2.3).
- 2) A temperature-controlled stirred water bath set at the appropriate temperatures for either 1 hour followed by 3 hour recovery at 37°C or for 0-2 hours followed by 1 hour of recovery at 37°C as previously described.

**A****B**

**Figure 2.3: Illustration of a thermal gradient bar.**

(A) The thermal gradient bar is composed of two circulating water baths at each end of an aluminium block. A temperature gradient naturally forms across the block allowing each column of receptacles to be a different temperature. (B) Measurement of temperatures across the gradient bar.



### 2.3.14 Extracellular HSP treatments.

Cells were incubated with either bovine Hsp70 or recombinant human Hsp72 which had been initially diluted in dH<sub>2</sub>O at a concentration of 0.5 mg/mL (bovine Hsp70) or PBS at a concentration of 0.1 mg/mL (human Hsp72). Cells were prepared for experimental treatments as in sections 2.3.10, 2.3.11 and 2.3.12. Bovine Hsp70 and human Hsp72 were made-up to the appropriate concentrations in phenol red-free 10 % RPMI (0-100 µg/mL) and added to cells for the appropriate time in a 37°C humidified incubator with 5 % CO<sub>2</sub>. Cells were transferred to 1.5 mL microcentrifuge tubes, centrifuged (500 g, 5 minutes at 25°C) and supernatant containing the treatments was removed and discarded.

### 2.3.15 Determination of apoptosis by Annexin V analysis by flow cytometry.

Annexin V is a 35-36 kDa Ca<sup>2+</sup> dependent phospholipid-binding protein that has a high affinity for the membrane lipid PS. In a normal population, PS is detectable as an internal membrane lipid, however during the events of early apoptosis, PS translocates to the membrane surface where Annexin V has the ability to bind. These circumstances enable Annexin V (which is FITC-conjugated) as a marker for early apoptotic events and in combination with PI, (which is PE-conjugated) allow for the distinguishing between viable, apoptotic, necrotic and secondary necrotic cells.

After treatment, U937 cells and leukocytes were washed with pre-warmed DPBS and centrifuged at 500 g for 5 minutes at 25°C. The supernatant was discarded and the cell pellet was re-suspended with 1X binding buffer. Cells were counted using trypan blue exclusion (Section 2.3.7) and the concentration was adjusted 1 x 10<sup>5</sup> cells/mL. The cell suspension was then incubated with 2.5 µL of Annexin V solution diluted in 50 µL of 1X binding buffer for 15 minutes at 25°C in the dark. After incubation, cells were treated with 2.5 µL of PI (PI concentration 100 µg/mL) solution diluted in 100 µL of 1X binding buffer and samples were analysed immediately by flow cytometry. Annexin V was detected at  $\lambda_{\text{ex}}$  496 nm/ $\lambda_{\text{em}}$  520 nm and PI was detected at 520 nm. Compensation controls were applied for each experiment and 10,000 events were recorded for each sample. Typically, positive (1 x 10<sup>5</sup> apoptotic cells - were prepared using camptothecin and 1 x 10<sup>5</sup> necrotic cells - were prepared by autoclaving in 10 % RPMI at 121°C for 20

minutes) and negative ( $1 \times 10^5$  untreated cells) controls were included in the analysis.

#### **2.3.16 Determination of apoptosis by caspase-3 fluorimetric assay.**

Caspase-3 is an enzyme activated by several apoptotic pathways. It is an effector caspase shown to cleave poly-(ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), topoisomerases, and protein kinase C. It is cleaved and activated by granzyme B which in turn activates a caspase proteolytic cascade resulting in apoptosis. For apoptosis detection, the EnzoLyte™ Rh110 Caspase-3 assay was used. It is optimised to detect the activation of down-stream caspase-3 activity using the synthetic peptide substrate (Z-DEVD)<sub>2</sub>-conjugated to the fluorogenic indicator Rh110 (rhodamine 110). Caspase-3 cleavage causes Z-DEVD<sub>2</sub>-Rh110 to generate the fluorophore Rh110 which has a bright green fluorescence that can be detected at  $\lambda_{\text{ex}}$  496 nm/  $\lambda_{\text{em}}$  520 nm using a fluorescence microplate reader.

After the relevant time period, U937 cells in 1.5 mL microcentrifuge tubes were centrifuged at 500 g for 5 minutes at 25°C, supernatant was removed and discarded and the cells were washed twice with 100  $\mu\text{L}$  of fresh 10 % RPMI. The cell pellet was re-suspended with 60  $\mu\text{L}$  of 10 % RPMI and was transferred to a black-sided clear-bottomed 384-well plate. A 20  $\mu\text{L}$  volume of caspase-3 substrate solution made according to manufacturer's instructions (1 mL of assay buffer, 40  $\mu\text{L}$  of 1 M DL-dithiothreitol (DTT) and 10.4  $\mu\text{L}$  of caspase-3 substrate) was immediately added to each test well and the plate was then incubated in the dark for 60 minutes at 25°C. Fluorescence intensity was measured at  $\lambda_{\text{ex}}$  496 nm/  $\lambda_{\text{em}}$  520 nm using a fluorescence microplate reader. Positive ( $3 \times 10^5$  apoptotic cells- prepared using camptothecin) and negative ( $3 \times 10^5$  untreated cells) controls were included in the analysis.

#### **2.3.17 Determination of apoptosis by caspase-3 analysis by flow cytometry.**

Active caspase-3 was also detected using a rabbit monoclonal antibody-FITC conjugated and directed against the active cleaved caspase-3. After treatment, human leukocytes were washed using 1 mL antibody wash buffer (DPBS

containing 5 % FBS), centrifuged at 500 *g* for 5 minutes at 25°C and the supernatant discarded. The leukocyte cell pellet was first labelled with a 4 µL volume of antibodies to surface CD markers (Table 2.1), with the exception of CD14 when a 1 µL volume was used. Fluorescent labelled antibodies to leukocyte markers CD3 (PE), CD14 (PE/Cy7) and CD15 (APC) were used to distinguish lymphocytes, monocytes and neutrophils respectively. Cells were incubated with these markers for 30 minutes at 4°C. Any unbound antibody was then washed with antibody wash buffer as previously described, and the supernatant was discarded. Cells were then fixed and permeabilized with 70 µL of fix/perm solution for 20 minutes on ice in the dark. Cells were washed and then centrifuged at 500 *g*, for 5 minutes at 25°C before the addition of 6.7 µL/well of a rabbit monoclonal anti-active-caspase-3-FITC conjugated antibody. Cells were incubated for 40 minutes on ice in the dark. After incubation, cells were washed twice with wash buffer, centrifuged at 500 *g*, for 5 minutes at 25°C and re-suspended with 100 µL of DPBS. Cells were then analysed by flow cytometry (Section 2.3.20), with 10,000 events recorded per sample. Typically, positive ( $3 \times 10^5$  apoptotic cells - were prepared using camptothecin) and negative ( $3 \times 10^5$  untreated cells) controls were included in the analysis and an isotype control of rabbit IgG1-FITC to evaluate background fluorescence. Compensation controls for each experiment were run initially and the settings were applied throughout the study.

### **2.3.18 Determination of necrosis by PI staining.**

A loss of cell membrane permeability and DNA fragmentation is considered to be a characteristic hallmark of necrosis. PI is a fluorescent dye that binds to DNA by intercalating with bases with a stoichiometry of one dye per 4 - 5 base pair and is excluded by intact cell membranes. Necrotic cells can be detected and quantified by staining of nuclear DNA by PI in a hypotonic buffer at  $\lambda_{\text{ex}}$  535 nm /  $\lambda_{\text{em}}$  617 nm.

U937 cells were treated as previously described (Section 2.3.12 and 2.3.13) and after the appropriate time period, were centrifuged at 500 *g* for 5 minutes at 25°C. The supernatant was removed and discarded then the cell pellet washed twice with 100 µL 10 % RPMI. The cell pellet was re-suspended with 50 µL of

10 % RPMI and was transferred to a black-sided clear-bottomed 96 well plate with 50  $\mu\text{L}$  of 5  $\mu\text{g/mL}$  working PI solution. The PI stock solution was maintained at  $-20^{\circ}\text{C}$  at a concentration of 100  $\mu\text{g/mL}$  reconstituted with sterile DPBS. A working solution of 5  $\mu\text{g/mL}$  diluted in sterile DPBS was prepared before each analysis. Plates were mixed and incubated in the dark for 20 minutes at  $25^{\circ}\text{C}$  and fluorescence intensity was measured,  $\lambda_{\text{ex}}$  535 nm /  $\lambda_{\text{em}}$  617 nm using a microplate reader. Included in the analysis were positive ( $3 \times 10^5$  necrotic cells – prepared by autoclaving in 10 % RPMI at  $121^{\circ}\text{C}$  for 20 minutes) and negative ( $3 \times 10^5$  untreated cells) controls and background controls of media only.

### **2.3.19 Determination of cellular viability by MTS assay.**

Cell viability was estimated using a colorimetric assay, CellTiter 96® AQueous One Solution Cell Proliferation assay, which was performed according to the manufacturer's instructions. Analysis is based upon the reduction of the tetrazolium salt, MTS and an electron coupling agent PES to a formazan product in the presence of metabolically active cells, which is then quantified by spectrophotometry. Reduction of MTS compound to formazan is detected by colour development at 490 nm using a microplate reader. The quantity of formazan produced is directly proportional to the number of metabolically active cells in culture.

After the relevant time period, U937 cells in 1.5 mL microcentrifuge tubes were centrifuged at 500  $g$  for 5 minutes at  $25^{\circ}\text{C}$ . The supernatant was removed and discarded and cells were washed twice with 100  $\mu\text{L}$  of fresh 10 % RPMI. The cell pellets were re-suspended with 100  $\mu\text{L}$  10 % RPMI and were transferred to a 96-well cell culture plate along with 20  $\mu\text{L}$  of thawed MTS solution per well. The plate was incubated in a humidified 5 %  $\text{CO}_2$  atmosphere at a temperature of  $37^{\circ}\text{C}$  for up to 3 hours. Absorbance was measured at 490 nm using a fluorescent microplate reader and a comparison with controls was performed. Controls included positive ( $3 \times 10^5$  necrotic cells – prepared by autoclaving in 10 % RPMI at  $121^{\circ}\text{C}$  for 20 minutes) and negative ( $3 \times 10^5$  untreated cells) controls and background controls of media only.

### **2.3.20 Flow cytometry.**

Flow cytometry was used to measure both CD markers and HSPs on the cell surface, membrane-bound or membrane-embedded and/or inside cells. After the relevant time period,  $3 \times 10^5$  cells in 1.5 mL microcentrifuge tubes were washed once with 1 mL antibody wash buffer (DPBS containing 5 % FBS) and centrifuged at 500 g for 5 minutes at 25°C. The supernatant was removed and discarded, the cell pellet was re-suspended in 150 µL of antibody wash buffer and the cells were transferred to a 96-well V-bottom plate. The cells were washed twice more in antibody wash buffer and the supernatant was removed and discarded.

#### **2.3.20.1 Cell surface detection of CD markers and HSPs.**

When analysing whole blood cell populations it is important to distinguish between leukocyte subtypes in order to investigate HSP surface and intracellular expression in each of them. For this purpose, antibodies to surface CD markers were used in conjunction with either anti-Hsp27: PE, anti-Hsp60: PE, anti-Hsp72: FITC, or anti-Hsp90: PE. The choice of fluorescent markers had to consider the spectral overlap and the capabilities of the FACSCanto instrument. The markers used are listed in table 2.1. Cells were counted using trypan blue exclusion (Section 2.3.7) and were adjusted to  $3 \times 10^5$  cells/mL. Cells were then centrifuged at 500 g for 5 minutes at 25°C, were re-suspended and incubated with the CD antibodies for 30 minutes at 4°C in the dark. Samples were then washed with antibody wash buffer, centrifuged at 500 g for 5 minutes at 25°C and the supernatant discarded. Cells were then incubated with either an anti-Hsp27: PE, anti-Hsp60: PE, anti-Hsp72: FITC or anti-Hsp90: PE antibody for 40 minutes at 4°C in the dark. Antibody quantities required can be found in table 2.2. The cells were washed twice with 1 mL antibody wash buffer, centrifuged (500 g, 5 minutes at 25°C) and the supernatant was removed and discarded. In order to discard in the analysis any necrotic cells, 2.5 µL of 100 µg/mL PI solution was added to the cell pellets and left for 5 minutes at 4°C. The cell pellets were re-suspended in 150 µL DPBS and were analysed immediately by flow cytometry. Cells negative to PI (viable cells) were gated and U937 or erythrocyte cell populations were gated using FSC/SSC, whilst leukocyte populations were gated using CD3, CD14, CD15 and FSC/SSC respectively to

discard cell debris. 10,000 events were recorded and compensation controls for each experiment were run initially and the settings were applied throughout the study.

#### **2.3.20.2 Intracellular HSP detection.**

Cells (leukocytes were first labelled with antibodies to surface CD markers as previously described in section 2.3.20.1 to distinguish relevant populations) were fixed and permeabilized with 70  $\mu$ L of fix/perm solution for 20 minutes at 4°C in the dark. Cells were washed twice with 1 mL antibody wash buffer, centrifuged at 500 *g* for 5 minutes at 25°C and the supernatant discarded. Cell suspensions were then incubated with 50  $\mu$ L/well of a 1:250 dilution of either anti-Hsp27: PE, anti-Hsp60: PE, anti-Hsp72: FITC or anti-Hsp90: PE antibody diluted in antibody wash buffer. Cells were incubated for 40 minutes at 4°C in the dark. Following incubation, cells were washed twice with 1 mL antibody wash buffer, centrifuged (500 *g*, 5 minutes at 25°C) and the supernatant discarded. Cells were re-suspended in 150  $\mu$ L of DPBS and analysed by flow cytometry. Cells were gated using FSC/SSC and leukocytes were gated using CD3, CD14, CD15 and FSC/SSC respectively to discard cell debris. 10,000 events were recorded and compensation controls for each experiment were run initially and the settings were applied throughout the study.

All values for flow cytometry were corrected for isotype controls, mouse IgG1-FITC and mouse IgG1-R-PE in order to evaluate background fluorescence.

**Table 2.1: Leukocyte subset classification using antibodies to surface CD markers.**

Leukocyte Subsets		Volume per $3 \times 10^5$ cells/mL
CD3-PE	T-lymphocytes	4 $\mu$ L
CD14-PE-Cy7	Monocytes	1 $\mu$ L
CD15-APC	Neutrophils	4 $\mu$ L

**Table 2.2: Volumes of HSP antibodies required for flow cytometric analyses.**

Antibody Name	Volume per $3 \times 10^5$ cells/mL
Anti-Hsp27: PE	50 $\mu$ L (1:250 dilution)
Anti-Hsp60: PE	50 $\mu$ L (1:250 dilution)
cm.Hsp70.1: FITC	5 $\mu$ L (1:5 dilution)
Anti-Hsp72: FITC	50 $\mu$ L (1:250 dilution)
Anti-Hsp90: PE	50 $\mu$ L (1:250 dilution)

### **2.3.21 Fluorescence microscopy.**

Fluorescence microscopy was used to visualise HSPs both on the cell surface and inside cells. Images were generated on a conventional fluorescence microscope, Eclipse TE2000-U microscope and photographed on a Hamamatsu CA4742-95 camera. Images were acquired at a magnification of x 40 or x 60 (Plan Fluro ELND 40x/0.60 or 60x/0.70) set up in FITC or TRITC-fluorescence mode maintained at 25°C. Image acquisition was performed using IPLAB 4.0 image analysis programme. After the relevant time period,  $1 \times 10^6$  cells in 1.5 mL microcentrifuge tubes were washed twice with 1 mL of pre-warmed DPBS, centrifuged at 500 g for 5 minutes at 25°C and the supernatant was removed and discarded.

#### **2.3.21.1 HSP cell surface detection.**

The cell pellets were fixed for 10 minutes with 3.7 % methanol free formaldehyde made in DPBS. Cells were washed three times in 1 mL DPBS, centrifuged (500 g, 5 minutes at 25°C) and the supernatant was discarded. A 50 µL volume of a 1:100 dilution (in DPBS containing 1 % BSA) of either an anti-Hsp27: PE, anti-Hsp60: PE, anti-Hsp72: FITC or anti-Hsp90: PE antibody was added to the cell pellet and incubated for 40 minutes at 4°C in the dark. After three washes with 1 mL of DPBS, cells were re-suspended in 5 µL of DPBS and were mounted onto a glass slide. A single drop of the anti-fade reagent ProLong® Gold containing DAPI (U937 cells and human leukocytes) or DABCO (human erythrocytes) was added to the cell suspension before the addition of a coverslip. Coverslips were sealed and the slides were left in the dark for 24 hours before observation under the fluorescence microscope.

#### **2.3.21.2 HSP intracellular detection.**

The cell pellets were fixed with 3.7 % methanol free formaldehyde and then permeabilized using 1 mL 0.1 % triton-X 100 in DPBS for 3-5 minutes at 25°C. Cells were washed twice with 1 mL DPBS, centrifuged (500 g, 5 minutes at 25°C) and the supernatant was discarded. 50 µL of anti-Hsp27: PE, anti-Hsp60: PE, anti-Hsp72: FITC or anti-Hsp90 PE conjugated antibodies (1:100 in DPBS containing 1 % BSA) were incubated with the cell pellets for 40 minutes at 4°C in the dark. After three washes in DPBS and re-suspending the cells in 5 µL



DPBS, cells were mounted onto glass slides as previously described in section 2.3.21.1 and examined under the fluorescence microscope.

### **2.3.22 Membrane extraction.**

Membrane extraction was performed using the Mem-PER® eukaryotic membrane protein extraction reagent kit. This kit allows the isolation of integral membrane proteins from cultured mammalian cells using a mild detergent.

In brief,  $5 \times 10^6$  isolated cells per sample were washed with PBS, centrifuged at 850 g for 2 minutes and supernatant discarded. Cells were lysed using 150  $\mu$ L of detergent A by pipetting up and down to obtain a homogenous cell suspension and were incubated for 10 minutes at RT with occasional vortexing. Lysed cells were then placed on ice whilst reagents B and C were mixed 1:2. 450  $\mu$ L of the mixed reagents were then added to each sample to solubilise the membrane proteins and were incubated on ice for 30 minutes with vortexing every 5 minutes. Samples were then centrifuged at 10,000 g for 3 minutes at 4°C, supernatant was transferred to a new tube and incubated for 20 minutes in a 37°C water bath to separate the membrane protein fraction. Samples were centrifuged at RT for 2 minutes at 10,000 g in order to isolate the hydrophobic fraction (bottom layer) from the hydrophilic fraction. The hydrophilic phase (top layer) was then carefully removed from the hydrophobic phase and placed in a new tube. Fractions were left on ice ready for membrane protein analysis. For SDS-PAGE, all samples were normalized to the volume of the hydrophilic fraction using detergent B diluted 4-fold with dH<sub>2</sub>O. Samples were diluted 2-5 fold to prevent band distortion.

### **2.3.23 Protein extraction.**

HSP expression was analysed in human leukocyte extracts following treatment with heat shock. Following treatment, cells were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 500 g for 5 minutes at 25°C. The supernatant was removed and the cells were then homogenised in 50  $\mu$ L of protein extraction buffer. Cells were then centrifuged at 13,500 g for 20 minutes at 25°C and the cell extract was collected.

### 2.3.24 SDS-PAGE electrophoresis.

In order to analyse membrane and cellular protein extracts rapidly sodium dodecyl sulphate – polyacrylamide electrophoresis (SDS-PAGE) was used. Following sample preparation (Section 2.3.22 and 2.3.23) the membrane and cell extract samples were diluted 1:1 with SDS reducing sample buffer and were denatured by heating to 85°C for 10 minutes. The samples were then allowed to cool for 10 minutes prior to use. Precision Plus® standards were also used, as molecular weight references and did not require any pre-treatments.

Solutions and buffers were prepared in advance (Section 2.2.7) and the Bio-Rad mini-protean® II electrophoresis apparatus was assembled according manufacturer's instructions and run according to the methods described by Laemmli (1970).

10 % separating gels were prepared by mixing 6.05 mL dH<sub>2</sub>O with 3.75 mL of 1.5 M Tris-HCl pH 8.8, 150 µL of 10 % SDS solution and 5.0 mL acrylamide-bis in a small side-arm flask. This solution was degassed under vacuum for 15 minutes before 50 µL of 10 % ammonium persulphate was added along with 7.5 µL of TEMED. The solution was quickly and carefully pipetted between the glass plates to prevent it mixing with air until 1 cm from the top of the inner plate. This was immediately overlaid with 500 µL of dH<sub>2</sub>O and left to polymerise for 45 minutes to 1 hour.

The dH<sub>2</sub>O was poured off and a 3 % stacking gel was prepared which acts to concentrate large sample volumes resulting in a better band resolution. 3.15 mL of dH<sub>2</sub>O was added to 1.25 mL of 0.5 M Tris-HCl pH 6.8, 50 µL of 10 % SDS solution and 500 µL acrylamide-bis in a small side-arm flask. This was degassed for 15 minutes under vacuum before the addition of 50 µL of 10 % ammonium persulphate and 5 µL TEMED. The 10-well combs were then placed into the apparatus and the stacking gel was carefully pipetted over the separating gel and left to polymerise for 30-45 minutes. After the time period, the well combs were removed by pulling them straight up slowly and gently and the gel apparatus was assembled. Clamp assemblies were snapped onto the inner cooling frame and this was lowered into the lower chamber of the Mini-Protean II cell. Electrode buffer

was added to the inner chamber and the outer tank so that the bottom 1 cm of the gel was immersed. A 25  $\mu\text{L}$  volume of each sample and a 5  $\mu\text{L}$  volume of Precision Plus® standard were then loaded onto the stacking gel and run at 150 V (constant voltage setting) for 45 minutes or until the bromophenol blue dye was within 2 mm of the bottom of the separating gel.

After electrophoresis was complete, the buffer was poured off and the gel was carefully removed and stained for total proteins (Section 2.3.25) or transferred to a Western blotting chamber and blotted onto nitrocellulose for probing with specific antibodies (Section 2.3.26).

### **2.3.25 SDS-PAGE total protein staining.**

In order to visualise the proteins on the SDS-PAGE gels, Imperial Protein Stain was used. After electrophoresis, the gels were removed from the apparatus and stacking gels were removed from the separating gels and were washed three times with  $\text{dH}_2\text{O}$  with gentle shaking. 20 mL of protein stain was then applied to each gel for 1 hour with gentle shaking. The stain was poured away and the gels were again washed with  $\text{dH}_2\text{O}$  in order to obtain a clear gel with intense blue stained protein bands. This was maximised by overnight washing with  $\text{dH}_2\text{O}$ . The gels were visualised and photographed using the Bio-Rad ChemiDoc imaging system.

**2.3.26 Western blotting.**

Following SDS-PAGE electrophoresis (Section 2.3.24), gels were subjected to electrophoretic transfer to nitrocellulose membrane by western blot using 10  $\mu$ L of pre-stained or colour markers and Bio-Rad mini-trans blot apparatus. Solutions and buffers were prepared in advance (Section 2.2.8) and pre-cut filter paper and sponges were soaked in transfer buffer. Following electrophoretic transfer, the stacking gel was removed from the gel using a razor blade. The Bio-Rad mini-trans blot apparatus was then assembled as described below from the bottom upwards:

PLASTIC (TOP)  
SPONGE  
FILTER PAPER MEMBRANE  
SEPARATING GEL  
NITROCELLULOSE MEMBRANE  
FILTER PAPER MEMBRANE  
FILTER PAPER MEMBRANE  
SPONGE  
BLACK PLASTIC (BOTTOM)

Care was then taken to place the nitrocellulose membrane on top of the gel to ensure no air bubbles were present and good contact was made. The transfer cassette was closed and placed within the Bio-Rad mini-trans blot apparatus with the cooling unit. The tank was filled with the remaining transfer buffer and run at 100 V for 1 hour. Following transfer the nitrocellulose blot was removed and immuno-stained using specific antibodies (Section 2.3.27).

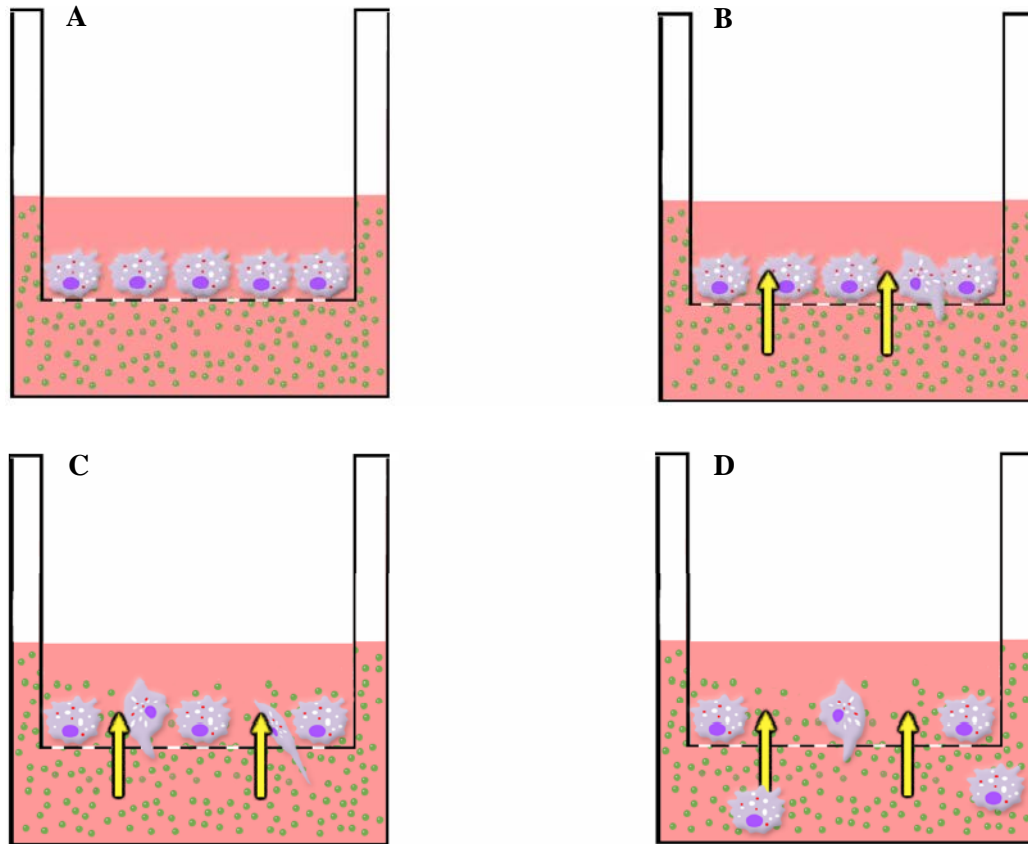
**2.3.27 Immuno-staining of Western blots.**

Buffers and solutions were prepared in advance (Section 2.2.8). Following Western blotting electro transfer, the blot was incubated for 1 hour at 25°C in blocking solution (1 % BSA in TBS). The blocking solution was then poured off and the blot was washed three times for 5 minutes per wash with 50 mL washing solution (TTBS). The blot was then incubated with a primary antibody in antibody buffer (1 % BSA in TTBS) and incubated for 1 hour at 25°C or

overnight at 4°C. The solution was removed and discarded and the blot was washed three times for 5 minutes per wash with 50 mL washing solution. The blot was then incubated with HRP labelled secondary antibody buffer for 1 hour at 25°C. The blot was washed a further five times with 50 mL washing buffer and then incubated with 2 mL of Supersignal West Pico or West Femto chemiluminescent substrate for 5 minutes. The blot was then visualised and photographed using the Bio-Rad ChemiDoc imaging system.

#### **2.3.28 Cell migration assay.**

Cells (100 µL/well) were added to the upper chamber of a modified Boyden chamber (HTS Transwell® permeable support system) and 200 µL of treatment was added to the lower chamber (Figure 2.4). Migration of cells was monitored at 2-4 hours at 37°C. Migrated cells were counted and viability tested by trypan blue exclusion (Section 2.3.7).



**Figure 2.4: Illustration of a modified HTS Transwell® permeable support system (Picture courtesy of Dr H. Elyse Ireland, University of Chester).**

(A) Cells are added to the top chamber and treatment is added to the bottom chamber. (B) As treatment diffuses through pores cells are activated and begin to migrate through the pores. (C) As the cells are further stimulated, more migration occurs. (D) Following treatment, the number of cells which have migrated through the pores are counted. The microporous membrane pore size was 3  $\mu\text{M}$  or 8  $\mu\text{M}$ . Neutrophils are approximately 10-12  $\mu\text{M}$ , lymphocytes are approximately 7-8  $\mu\text{M}$ , monocytes are approximately 14-17  $\mu\text{M}$ , whilst U937 macrophages are approximately 15-20  $\mu\text{M}$ .

### 2.3.29 HSP receptor analysis.

Flow cytometry was used to measure both Hsp70 and Hsp72 receptors on the cell surface. The presence of potential HSP receptors on cell membranes was tested in two ways:

1) Competitive inhibition experiments using known receptor antagonists: malBSA, fucoidan and poly (I) or Annexin V for PS analysis. Briefly, 100  $\mu$ L of 10  $\mu$ g/mL dilution (in antibody wash buffer) of malBSA, fucoidan, or poly (I) was added to 100  $\mu$ L of  $3 \times 10^5$  cells/mL for 1 hour at 37°C. Or, 2.5  $\mu$ L of Annexin V solution diluted in 50  $\mu$ L of 1X binding buffer was added to  $1 \times 10^5$  cells/mL for 15 minutes at 37°C. After treatment, cells were washed twice with 1 mL of antibody wash buffer, centrifuged (500 g, 5 minutes at 25°C) and the supernatant was removed and discarded. Cells were then incubated for 1 hour with 10  $\mu$ g/mL human Hsp72 or bovine Hsp70 at 37°C. Following treatment any unbound protein was washed away with two washes of 1 mL of antibody wash buffer, centrifuged at 500 g for 5 minutes at 25°C and the supernatant was removed and discarded. The cell pellet was re-suspended with 150  $\mu$ L of DPBS and HSP surface binding was determined immediately by flow cytometry using the methods previously described in section 2.3.20.

2) Using receptor specific antibodies to LOX-1 and SR-A1. Briefly, Cells were incubated with 100  $\mu$ L of a 1:10 or 1:50 dilution of anti-LOX-1 or anti-SR-A1 diluted in antibody wash buffer for 1 hour at 37°C, followed by the addition of secondary R-PE or FITC conjugated antibodies (100  $\mu$ L/well of a 1:10 or 1:50 dilution in antibody wash buffer) for 1 hour at 37°C. After treatment, cells were washed twice with 1 mL of wash buffer, centrifuged (500 g, 5 minutes at 25°C) and supernatant was removed and discarded. Cells were then processed for flow cytometry analysis (Section 2.3.20). All values for flow cytometry were corrected for isotype controls, mouse IgG1-R-PE and goat IgG-R-PE or FITC in order to evaluate background fluorescence.

### 2.3.30 Preparation of maleylated bovine serum albumin (malBSA).

Maleylated BSA was made according to published protocols (Takata, Horiuchi & Morino, 1989). In brief, 20 mg of BSA was dissolved in 10 mL of 0.1 M sodium

pyrophosphate buffer (pH 9.0) followed by dropwise addition of ice cold 1 M maleic anhydride in dioxane, total volume 750  $\mu$ L. The pH was maintained at 9.0 using NaOH solution. After incubation on ice for 5 minutes, the mixture was dialyzed extensively at 4°C for 24 hour against 0.15 M NaCl and 20 mM sodium phosphate buffer (pH 7.4). The concentration of malBSA was determined using a Bio-Rad protein assay (Section 2.3.31) to be 0.7 mg/mL.

### **2.3.31 Determination of protein concentration of samples.**

The total protein content of malBSA was determined using a Bio-Rad protein assay which is a modified version of the well documented Bradford Binding Assay (Bradford, 1976). It is a simple and accurate colorimetric assay for determining the concentration of solubilised protein which involves the addition of an acidic dye to a protein solution and measurement at 595 nm using a microplate reader. The assay is based on the observation that the absorbance maximum for an acidic solution of coomassie brilliant blue dye G-250 which binds to amino acid residues, shifts from 465 nm to a minimum of 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilise the anionic form of the dye, causing a visible colour change from brown to blue. Comparison to a standard curve provides a relative measurement of protein concentration.

Dye reagent was prepared by diluting one part dye reagent concentrate with four parts distilled, deionised water. It was filtered through a Whatman #1 filter paper to remove particles and kept at room temperature for a maximum of two weeks. Protein standards (0.05 mg/mL-0.5 mg/mL) were prepared using BSA diluted in DPBS. 10  $\mu$ L of the standards, zero control and samples were added in triplicate into a clean, dry non-binding 96-well microtitre plate followed by the addition of 200  $\mu$ L of diluted dye reagent solution. The plate was then incubated at 25°C with shaking for 5 minutes. After 15 minutes at room temperature, the absorbance of standards and unknown samples was measured at 595 nm.

### **2.3.32 Measuring erythrocyte lysis.**

Erythrocytes were centrifuged (350 g, 10 minutes at 25°C), transferred to a 96-well non-binding plate (100  $\mu$ L/well) and haemoglobin leakage from



erythrocytes was determined by absorbance at 550 nm, according to previous studies (de Brui et al. 2005; Motta et al. 1998), using a microplate reader. Absorbance values for test samples were then converted to percentage lysis using the following equation:

$$\text{Lysis (\%)} = \frac{\text{Absorbance}}{\text{Absorbance Max}} \times 100$$

For determination of 100 % lysis (absorbance maximum), an equal volume of dH<sub>2</sub>O was added to isolated erythrocytes and mean maximal absorbance was determined as previously described.

### **2.3.33 Preparation of NaCl for experiments.**

NaCl was reconstituted in 100 mM dH<sub>2</sub>O and allowed to dissolve for 5 minutes. NaCl was stored at 4°C for a maximum of 3 months.

### **2.3.34 Preparation of uric acid for experiments.**

Uric acid was reconstituted in 2 mM ammonium hydroxide at a molar ratio of ammonium hydroxide to uric acid of 1.7:1 and allowed to dissolve for 5 minutes. Uric acid was stored at -20°C for a maximum of 3 months.

### **2.3.35 Preparation of PMA for experiments.**

PMA was reconstituted in DMSO at 1 mg/mL and allowed to dissolve. PMA was then further diluted in 10 % HI-RPMI to give a 1 µg/mL stock solution. PMA was stored at -20°C for a maximum of 3 months.

### **2.3.36 Preparation of Brij®-98 for experiments.**

Brij®-98 was reconstituted in DPBS at 1 mg/mL and allowed to dissolve. Brij®-98 was then further diluted in 10 % HI-RPMI to give a 10 % stock solution. Brij®-98 was stored at 4°C for a maximum of 3 months. Brij®-98 is a nonionic surfactant that contains alkyl and polyoxyethylene, it is used to isolate fractions of the lipid membrane including sphingolipids and cholesterol by dissolving the

fluid membrane whilst isolating and leaving the lipid fractions intact (Bausero et al. 2005; Gastpar et al. 2005).

#### **2.3.37 Preparation of LPS for experiments.**

LPS from *E. coli* 0111:B4 was reconstituted in 10 % HI-RPMI at 1 mg/mL and allowed to dissolve. LPS was then further diluted in 10 % HI-RMPI to give a 1 µg/mL stock solution. LPS was stored at -20°C for a maximum of 3 months.

#### **2.3.38 Preparation of FMLP for experiments.**

FMLP was reconstituted in ethanol at 2 mg/mL (5 mM) and allowed to dissolve. FMLP was then further diluted in 10 % HI-RPMI to give a 5 µM stock solution. FMLP was stored at -20°C for a maximum for 3 months.

#### **2.3.39 Determination of TNF- $\alpha$ concentration by ELISA.**

The concentration of TNF- $\alpha$  was determined using a commercially available enzyme linked immunosorbant assay (ELISA).

The ELISA is an indirect ‘sandwich’ assay which utilises a plate bound antibody (anti-TNF- $\alpha$ ) with specificity for a capture antigen (TNF- $\alpha$ ) in an unknown sample. After immobilization of the antigen to a solid surface of the ELISA plate, a biotinylated detector antibody is added which forms a complex with the antigen followed by the addition of a secondary peroxidase-labelled avidin antibody. Between each step the plate is washed using an automated plate washer with ELISA wash solution to remove any proteins or antibodies that are not specifically bound. After this final step the plate is developed by adding TMB substrate which converts the peroxidase into a visible colorimetric form which is measured using a microplate reader at 450 nm, providing absorbance values which indicate the amount of antigen in the sample. Using known concentrations of TNF- $\alpha$  standards the concentration of unknown samples can be quantitatively determined.

An ELISA plate was coated with 100 µL/well of capture antibody in coating buffer at 1:250 dilution and incubated at 4°C overnight. The plate was washed five times with 250 µL/well ELISA wash buffer (PBS, pH 7.2 plus 0.05 % (v/v)

Tween<sup>®</sup>20 and 0.01 % (w/v) thimerosal) using an automated plate washer with a soak time between each wash of 1 minute. The plate was blotted dry and all wells were blocked with 200 µL/well of 1X assay diluent and incubated for 1 hour at 25°C. The plate was then washed and blotted dry as previously described. Standards (0-500 pg/mL) were prepared by diluting TNF-α stock in 1X assay diluent. Samples were either applied directly to the plate or diluted, if required, in 1X assay diluent. Standards and samples were applied to the plate at 100 µL/well and incubated overnight at 4°C. The plate was then washed and dried as before and 100 µL/well of biotinylated detector antibody diluted 1:250 in 1X assay diluent was added and incubated at 25°C for 1 hour. The plate was then washed and dried as before and 100 µL/well of peroxidase labelled avidin diluted 1:250 in 1X assay diluent was added and incubated at 25°C for 1 hour. The plate was then washed seven times with a soak time of 1 minute between washes and blot dried. TMB substrate solution was then added to the plate at 100 µL/well and incubated for 15 minutes with shaking before the addition of 1 M orthophosphoric acid 50 µL/well to stop the reaction. The plate was then read at 450 nm using a plate reader.

Absorbance values were then converted to TNF-α concentrations and concentration of unknown samples determined using Prism<sup>™</sup> 4.03 software.

#### **2.3.40 Determination of IL-10 concentration by ELISA.**

The concentration of IL-10 was determined using a commercially available indirect sandwich ELISA.

An ELISA plate was coated with 100 µL/well of capture antibody in coating buffer at 1:250 dilution, the plate was then sealed and incubated at 4°C overnight. The plate was washed five times with 250 µL/well ELISA wash buffer (PBS, pH 7.2 plus 0.05 % (v/v) Tween<sup>®</sup>20 and 0.01 % (w/v) thimerosal) with a soak time between each wash of 1 minute. The plate was blotted dry and all wells were blocked with 200 µL/well of 1X assay diluent and incubated for 1 hour at 25°C. The plate was washed and blotted dry as previously described. Standards (0-200 pg/mL) were prepared by diluting IL-10 stock in 1X assay diluent. Samples were either applied directly to the plate or diluted, if required, in 1X

assay diluent. Standards and samples were applied to the plate at 100  $\mu$ L/well and incubated overnight at 4°C. The plate was then washed and blotted dry as previously described and 100  $\mu$ L/well of a 1:250 dilution of biotinylated detector antibody in 1X assay diluent was added and incubated at 25°C for 1 hour. The plate was then washed and dried as before and 100  $\mu$ L/well of a 1:250 dilution peroxidase labelled avidin in 1X assay diluent was added and incubated at 25°C for 1 hour. The plate was then washed seven times with a soak time of 1 minute between washes and blotted dry. TMB substrate solution was then added to the plate (100  $\mu$ L/well) and incubated for 15 minutes with shaking before the addition of 1 M orthophosphoric acid (50  $\mu$ L/well) to stop the reaction. The plate was then read at 450 nm using a plate reader.

Absorbance values were then converted to IL-10 concentrations and concentration of unknown samples determined using Prism™ 4.03 software.

#### **2.3.41 Determination of Hsp72 concentration by ELISA.**

The concentration of Hsp72 was determined using an in house developed ELISA.

A Nunc Immuno Maxisorp 96-well plate was coated with 100  $\mu$ L/well of capture antibody in 0.1 M sodium bicarbonate buffer at 2  $\mu$ g/mL (1:500 dilution) and incubated at 4°C overnight. The plate was washed three times with 350  $\mu$ L/well ELISA wash buffer (PBS, pH 7.2 plus 0.05 % (v/v) Tween®20 and 0.01 % (w/v) thimerosal) using an automated plate washer with a soak time between each wash of 1 minute. The plate was blotted dry and all wells were blocked with 300  $\mu$ L/well of 0.5 % BSA (w/v) in PBS and incubated for 1 hour at 25°C. The plate was then washed and blot dried as previously described. Standards (0-50 ng/mL) were prepared by diluting Hsp72 stock in HI-RPMI. Samples were applied directly to the plate. Standards and samples were applied to the plate at 100  $\mu$ L/well and incubated for 2 hours at 37°C. The plate was washed five times and dried as before and 100  $\mu$ L/well of detector antibody diluted 1:1000 with 0.5 % BSA (w/v) in wash buffer was added and incubated at 37°C for 1 hour. The plate was then washed and blot dried as previously described. 100  $\mu$ L/well of an anti-mouse IgG peroxidase-labelled antibody diluted 1:1250 with 0.5 % BSA in wash buffer was then added and incubated at 25°C for 1 hour. The plate

was washed seven times with a soak time of 1 minute between washes and blot dried. TMB substrate solution was then applied to the plate (100  $\mu$ L/well) and incubated for 30 minutes with shaking before the addition of 1 M orthophosphoric acid (50  $\mu$ L/well) to stop the reaction. The plate was then read at 450 nm using a plate reader.

Absorbance values were then converted to Hsp72 concentrations and concentration of unknown samples determined using Prism™ 4.03 software.

#### **2.3.42 Statistical analyses.**

All statistical analyses were performed using the statistical software package PRISM™ version 4.03 (GraphPad Software Inc., San Diego). All data are expressed as the mean  $\pm$  standard error of the mean (SEM). The number of replicates (n) are shown in the figure legends where appropriate. Statistical comparisons were made using the unpaired Student's two-tailed t-test (single variables) and one and two-way ANOVA (time or concentration effects) with appropriate *post hoc* multiple comparisons tests, Dunnett's or Bonferroni's tests unless otherwise stated in the figure legends.

## **Chapter 3**

### **The effects of heat shock treatment on cell viability and, HSP expression and movement, in U937 cells and human leukocytes.**

#### **3.1 Introduction**

Intracellular HSPs are necessary for both normal cellular functions (Hartl & Hayer-Hartl, 2002) and for the protection of cells from various environmental stressors, such as increased temperature stress (Morimoto & Santoro, 1998), which would otherwise impair cellular functions, damage cellular structures and lead to apoptotic or necrotic cell death (Milleron & Bratton, 2006). It has been reported that heat stress induces an array of different intracellular HSPs, among which Hsp72 is the most abundant (Garrido et al. 2001; Schneider et al. 2002). HSPs allow cells to adapt to gradual changes in their environment, preventing protein denaturation and irreversible aggregation (Bukau & Horwich, 1998; Morimoto & Santoro, 1998; Hartl & Hayer-Hartl, 2002). It appears HSPs can be induced by apoptotic or necrotic stimuli and on the other hand can confer resistance to these stimuli, therefore the events of cell stress and death are suggested to be inter-linked. Several studies have indicated a role for intracellular HSPs in cyto-protection through functioning as anti-apoptotic agents which interfere with apoptotic pathways at multiple stages (Creagh et al. 2000; Mosser et al. 2000; Garrido et al. 2001; Jäättelä, 2004; Nylandsted et al. 2004; Ran et al. 2004; Stankiewicz et al. 2005). In contrast, a number of studies have also implicated intracellular HSPs induced by environmental stresses in the events leading to cell death through functioning as pro-apoptotic agents (Wei et al. 1995; Galea-Lauri et al. 1996; Liossis et al. 1997).

In addition to the intracellular expression of HSPs, there is also recurrent evidence suggesting the unusual localisation of HSPs on the cell surface (membrane-bound or embedded) of viral or bacterial-infected cell lines (Di Cesare et al. 1992; Chouchane et al. 1994) and on a variety of tumour cell lines (Multhoff et al. 1995; Botzler et al. 1996; Kleinjung et al. 2003; Korbelik et al. 2005; Steiner et al. 2006; Cid et al. 2008; Gehrmann et al. 2008). These proteins, for example Hsp72, have been found to exhibit immunological potential through stimulating NK cells to attack tumour cells which have part of the Hsp72 C-

terminal domain expressed on the extracellular surface (Botzler et al. 1998; Multhoff et al. 1999; Gastpar et al. 2005). To date however, there is no conclusive evidence of the cell surface expression of HSPs on normal or stressed human peripheral blood cells. Although the immunological role of HSPs on the cell surface of tumour cells are apparent, the transport of intracellular HSPs to the cell surface remains inconclusive, because HSPs are known to lack a consensual secretory signal that would usually enable translocation. However, as previously described, a major role of intracellular HSPs are as molecular chaperones, therefore it has been proposed that HSPs are transported to the cell surface in combination with other proteins which do possess trans-membrane domains (Multhoff, 2007). Furthermore, there is also evidence which suggests that intracellular HSPs are transported to the cell surface through a direct interaction with lipid components, either constitutively or following a physiological insult (Guzhova et al. 2001; Barreto et al. 2003; Hunter-Lavin et al. 2004b; Bausero et al. 2005; Tytell; 2005). Indeed, the cell surface expression of certain HSPs have been shown to be temporarily associated with membrane receptors such as TLR4/CD14 clusters in lipid rafts (Triantafilou et al. 2001b; Triantafilou & Triantafilou, 2002b), where a reported function is to stabilise the lipid membrane during stress (Tsvetkova et al. 2002), and PS on tumour cells (Arispe et al. 2004; Schilling et al. 2009). It has been speculated, following an association between intracellular Hsp72 and PS on the inner side of the plasma membrane, a 'flipping' mechanism occurs whereby Hsp72 is transported from the inside of the cell to the outer plasma membrane leaflet (Arispe et al. 2004; Vega et al. 2008). However, in contrast to stabilising the lipid membrane, the association of cell surface HSPs with external PS, an early indicator of apoptosis, is thought to be associated with a decreased viability of the cell (Arispe et al. 2004). This has also been demonstrated by several other investigators (Poccia et al. 1996; Sapozhnikov et al. 1999; Sapozhnikov et al. 2002; Feng et al. 2003; Gross et al. 2003b; Korbelik et al. 2005), who suggested an increased level of cell surface expressed HSPs correlated with a high susceptibility of the cells to apoptosis.

### **3.1.1 Aims**

The aim of this chapter is to determine the effects of *in vitro* elevated temperature stress in U937 monocytes and human leukocytes. It also aims to explore the up-regulation, localisation and movement of HSPs, and investigates the role of HSPs in apoptosis and necrosis through examining a potential relationship between heat-induced cell death and HSP translocation to the cell surface.



## **3.2 Methods**

All preparations and cell culture experiments were performed using aseptic technique in a class II tissue culture hood.

### **3.2.1 Preparation of cells for experimental treatment.**

Human monocytic U937 cells were prepared as in section 2.3.12. Human leukocytes were isolated and prepared for treatment as in section 2.3.10.

### **3.2.2 Time course experiments with heat shock.**

Two *in vitro* heat treatment experiments were performed using:

- 1) A thermal gradient bar set to 37.7°C-46.2°C for 0-6 hours then transferred to a 37°C incubator with 5 % CO<sub>2</sub> for 1 hour recovery.
- 2) A temperature-controlled stirred water bath set to 37°C or 42°C for 1 hour followed by 3 hours of recovery at 37°C.

### **3.2.3 Determination of apoptosis.**

Apoptosis was measured by the microplate based caspase-3 fluorimetric assay (Section 2.3.16), or by flow cytometry analysis of Annexin V (Section 2.3.15) and caspase-3 (Section 2.3.17).

### **3.2.4 Determination of necrosis.**

Necrosis was determined by PI staining (Section 2.3.18).

### **3.2.5 Determination of cell viability and proliferation.**

Cell viability was estimated using a colorimetric assay, CellTiter 96® AQueous One Solution Cell Proliferation assay (Section 2.3.19).

### **3.2.6 Measurement of surface and whole cell HSPs.**

Flow cytometry and fluorescence microscopy were used to measure HSPs on the cell surface (membrane-bound or embedded on non-permeabilized cells) or on/inside (permeabilized cells) U937 cells and human leukocytes (Sections 2.3.20 and 2.3.21).

Cells were incubated with anti-HSP antibodies for 40 minutes at 4°C in the dark.

1) Flow Cytometry: Anti-Hsp27: PE, anti-Hsp60: PE, anti-Hsp72: FITC or anti-Hsp90: PE were applied to cells at a 1:250 dilution 50  $\mu$ L/sample. Anti-cmHsp70.1: FITC was applied to cells at a 1:5 dilution 5  $\mu$ L/sample.

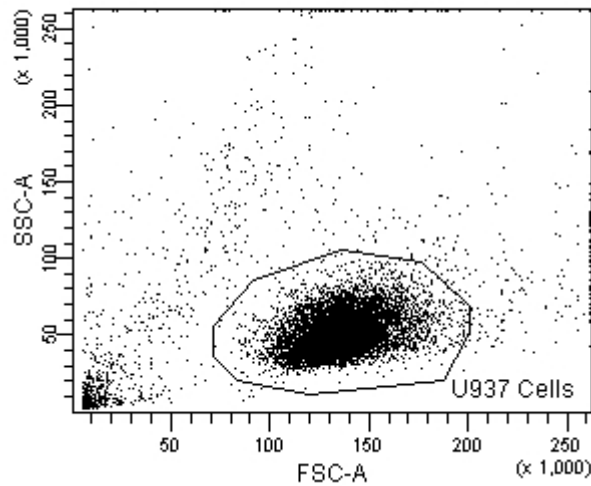
2) Fluorescence Microscopy: Anti-HSPs: FITC or PE were applied to cells at a 1:100 dilution 50 $\mu$ L/sample.

### **3.2.7 Detection of HSPs.**

Human leukocyte membranes were prepared for analysis using methods described in section 2.3.22. HSPs were visualised following SDS-PAGE (section 2.3.24) and Western blotting (section 2.3.26).

### 3.3 Results

The first part of the study analysed the effects of elevated temperature stress in human monocytic U937 cells. In both control and heat treated samples, U937 cells were identified using forward and side scatter (FSC/SSC) on the flow cytometer (Figure 3.1). Cell viability was determined using a microplate colorimetric assay (Figure 3.4A), whilst apoptotic and necrotic cell death were quantitatively measured by flow cytometry analysis of Annexin V (Figures 3.2-3.3) or microplate based assays measuring caspase-3 and PI on a fluorescence microplate reader (Figure 3.4B-C).



**Figure 3.1: Identification of human monocytic U937 cells using FSC/SSC on the flow cytometer.**

$3 \times 10^5$  cells/mL U937 cells were gated using FSC/SSC respectively. Data are representative of three independent experiments and 10,000 events were recorded.

### **3.3.1 Effect on cell viability and the induction of apoptotic or necrotic cell death following treatment of U937 cells with heat stress.**

The effect on cell viability and the induction of apoptosis and necrosis by different temperature treatments are presented in Figures 3.2-3.4.

U937 cells at a concentration of  $3 \times 10^5$  cells/mL were incubated at increasing temperatures of 37.7°C-46.2°C for six hours, followed by one hour recovery at 37°C. As shown in the dot blots presented in figure 3.2 and the temperature response curve in figure 3.3, when U937 cells were incubated at temperatures above 37.7°C, cell viability was significantly reduced ( $P < 0.001$ ) as revealed by an increase in apoptotic and necrotic cell death compared to control cells. A rapid increase in the number of apoptotic cells was observed at 40.8°C, approximately 70 % ( $P < 0.001$ ) compared to control cells, which then declined with increasing temperatures. Apoptosis decreased by 17 % from 72 % ( $P < 0.001$ ) at 42.7°C, by 22 % from 55 % ( $P < 0.001$ ) at 44.4°C and by 20 % from 33 % ( $P > 0.05$ ) at 46.2°C. No other significant decrease was observed. The decline in apoptotic cell death was followed by a significant increase in the number of necrotic cells ( $P < 0.001$ ) compared to control, approximately 25 % at 42.7°C ( $P < 0.001$ ). At temperatures lower than this (40.8°C), a non-significant increase was observed. Necrotic cell death increased with increasing temperatures, approximately 40 % at 44.4°C ( $P < 0.001$ ), which increased to approximately 96 % at 46.2°C ( $P < 0.001$ ) (Figures 3.2-3.3).

Time course experiments exposing U937 cells to elevated temperatures seen in figures 3.2-3.3 over a time period of six hours revealed a significant decrease in U937 cell viability ( $P < 0.001$ , Figure 3.4A), a significant increase in the number of apoptotic U937 cells as measured by caspase-3 activity ( $P < 0.001$ , Figure 3.4B), and a significant increase in necrosis due to an increase in PI fluorescence when compared to control cells ( $P < 0.001$ , Figure 3.4C).

#### **3.3.1.1 Effect on cell viability.**

There was a significant reduction of U937 cell viability after one hour incubation at 44.4°C compared to control cells ( $P < 0.001$ , Figure 3.4A). At temperatures lower than this (40.8°C and 42.7°C) a non-significant effect after one hour

incubation was observed (Figure 3.4A). As the length of exposure to elevated temperatures increased, the viability of U937 cells was significantly reduced compared to control cells ( $P < 0.001$ , Figure 3.4A), and after six hours of incubation all temperatures resulted with a significant decline of U937 cell viability compared to control cells ( $P < 0.001$ , Figure 3.4A), which was consistent with the results obtained in figures 3.2-3.3.

#### **3.3.1.2 Induction of apoptosis in U937 cells.**

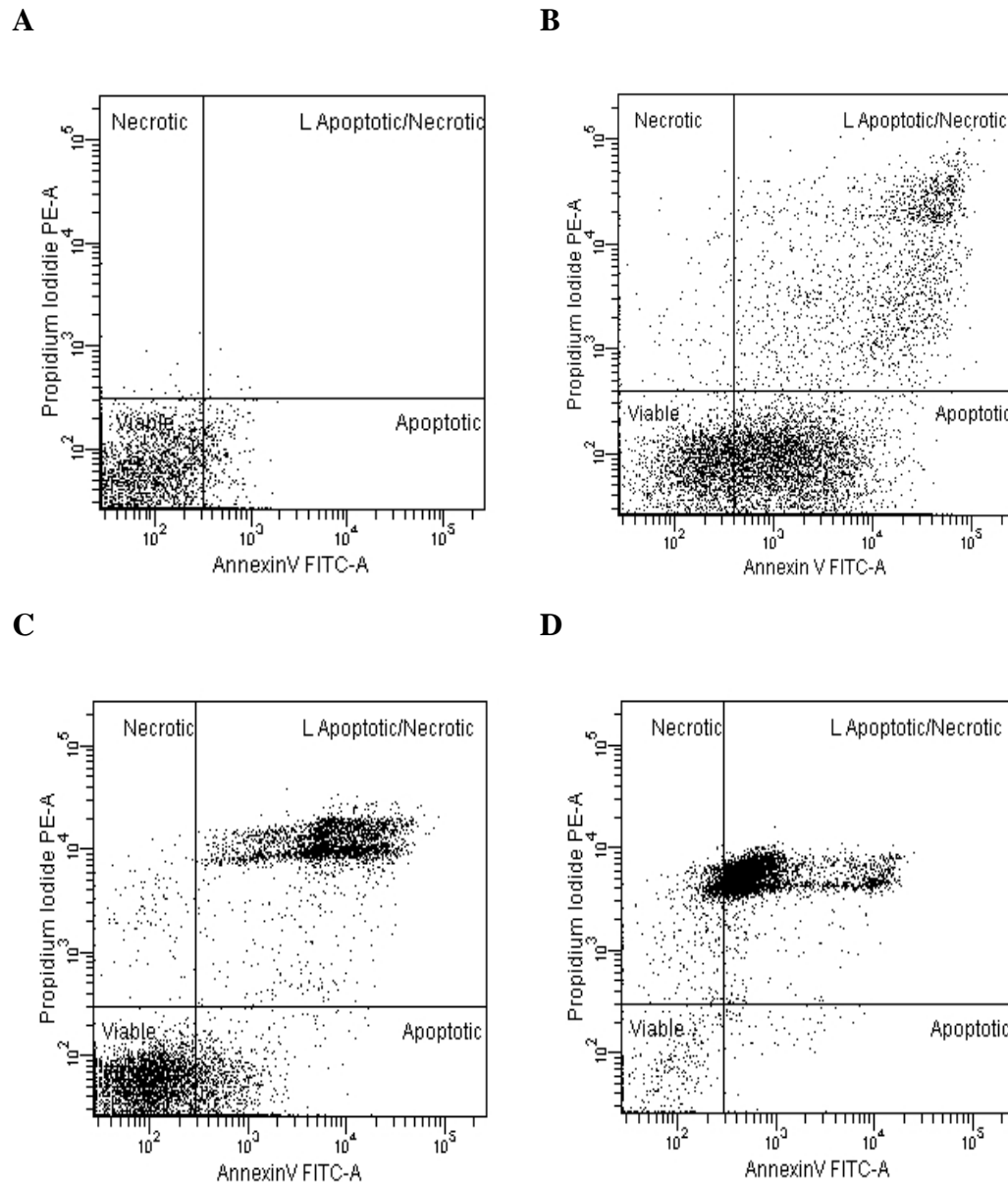
A one hour exposure of U937 cells to a 42.7°C heat shock led to a significant increase in apoptotic cell death compared to control cells ( $P < 0.001$ , Figure 3.4 B), however at this temperature a non-significant decline in cell viability was observed (Figure 3.4A). At the lower temperature of 40.8°C a non-significant increase in apoptotic cell death was observed after a one hour incubation (Figure 3.4B), however at the elevated temperatures of 44.4°C and 46.2°C, both were significantly different from control cells ( $P < 0.001$ , Figure 3.4B). Apoptosis was revealed to increase over time ( $P < 0.001$ , Figure 3.4B) and a peak of U937 cell apoptosis was observed at 40.8°C after a three hour exposure ( $P < 0.001$ , Figure 3.4B), but reduced thereafter up to six hours which was consistent with previous findings (Figures 3.2-3.3). Following incubation of U937 cells at the elevated temperatures of 44.4°C and 46.2°C there was also a significant decline in apoptosis from two hours (2-fold and 3-fold decrease) up to six hours (4-fold and 5-fold decrease) leading to a non-significant difference from control cells (Figure 3.4B).

#### **3.3.1.3 Induction of necrosis in U937 cells.**

Exposing U937 cells to increasing temperatures over a time period of six hours provided evidence for necrotic cell death in U937 cells compared to control cells ( $P < 0.001$ , Figure 3.4C). After a one hour incubation at 44.4°C a significant increase in necrosis was observed ( $P < 0.001$ , Figure 3.4C), however at temperatures lower than this (40.8°C and 42.7°C) there was a non-significant difference from control (Figure 3.4C). Similar to apoptosis, necrosis significantly increased with elevated temperatures over time compared to control cells ( $P < 0.001$ , Figure 3.4C) and after six hours of incubation all temperatures resulted with a significant increase in U937 necrotic cell death compared to control cells

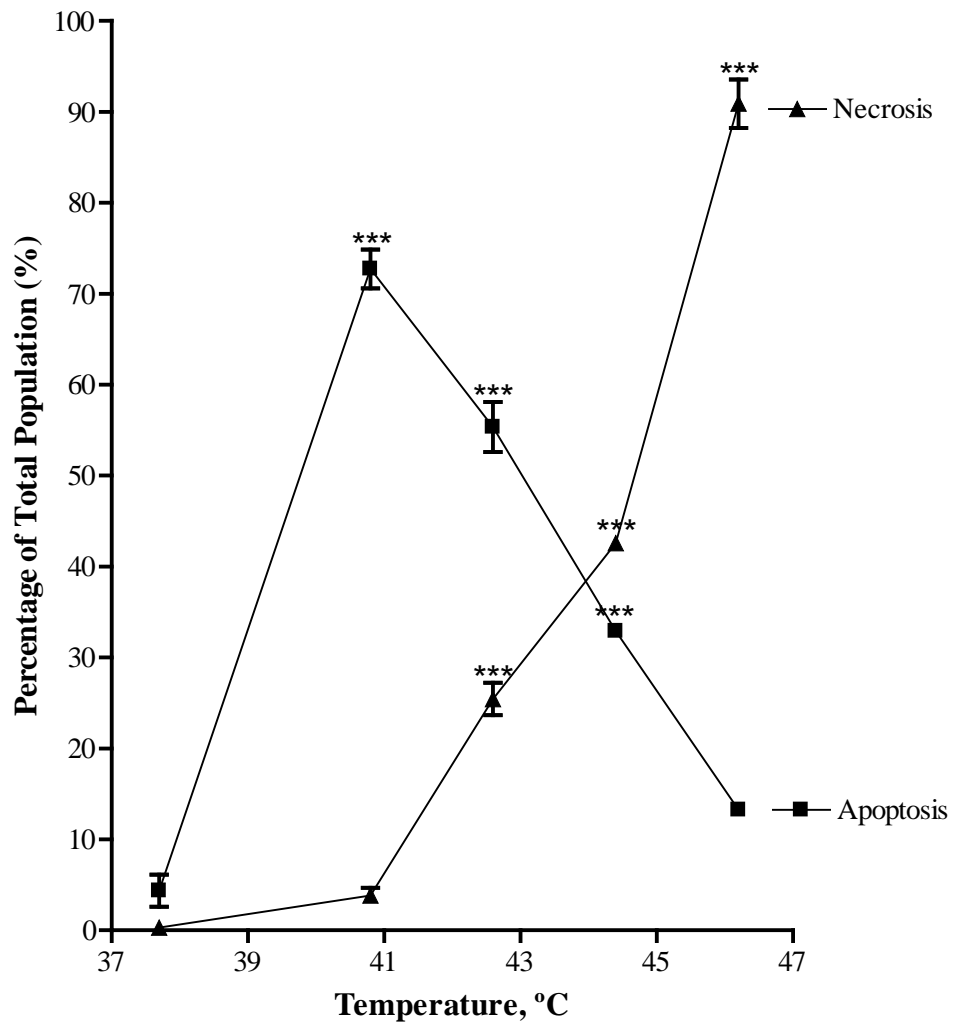
( $P < 0.001$ , Figure 3.4C), which was consistent with the results obtained in figures 3.2-3.3.

These data conclude that human monocytic U937 cells undergo apoptosis after a one hour exposure to 42.7°C heat shock (approximately 30 %), whilst a peak in apoptosis was observed at the specific time and temperature of a three hour exposure to 40.8°C (approximately 80 %). Increasing the temperature results in the mode of cell death switching from apoptosis to necrosis where an increase in PI fluorescence was detected along with a decline in U937 cell viability.



**Figure 3.2: Annexin V and PI assay performed on U937 cells when incubated for 6 hours at 37.7°C-44.4°C and following 1 hour recovery at 37°C.**

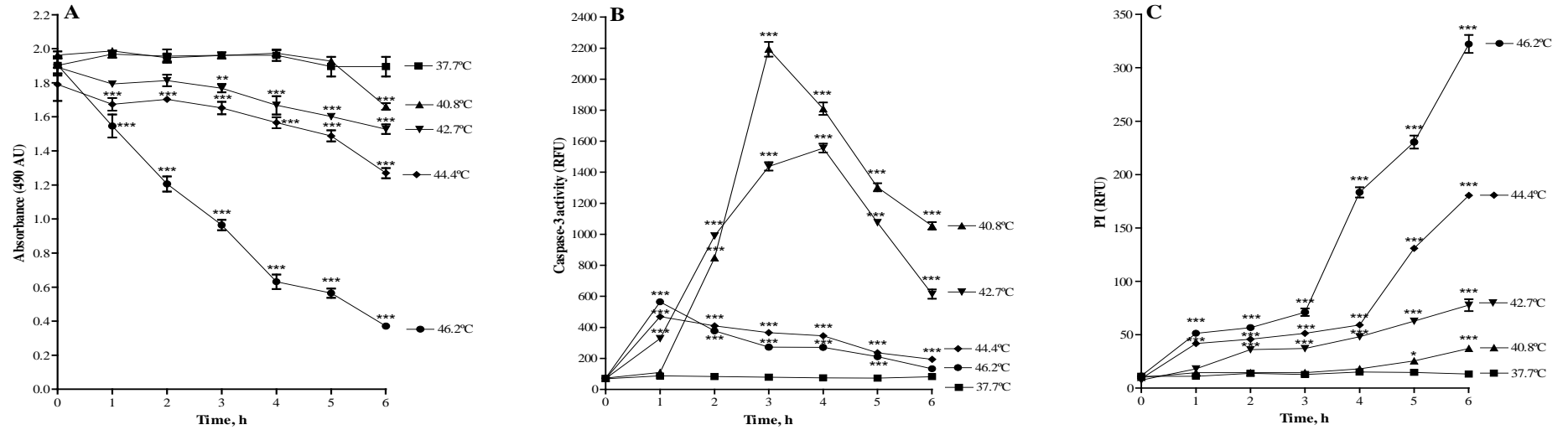
U937 cells were gated using FSC/SSC respectively. (A) Control 37.7°C (6 hours) (B) Heat Shock 40.8°C (6 hours), (C) Heat Shock 42.7°C (6 hours) and (D) Heat Shock 44.4°C (6 hours). Data are representative of three independent experiments and 10,000 events were recorded.



**Figure 3.3: Percentage of apoptotic and necrotic U937 cells in response to 6 hour heat shock treatment at 37.7°C-46.2°C and following 1 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from 37.7°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



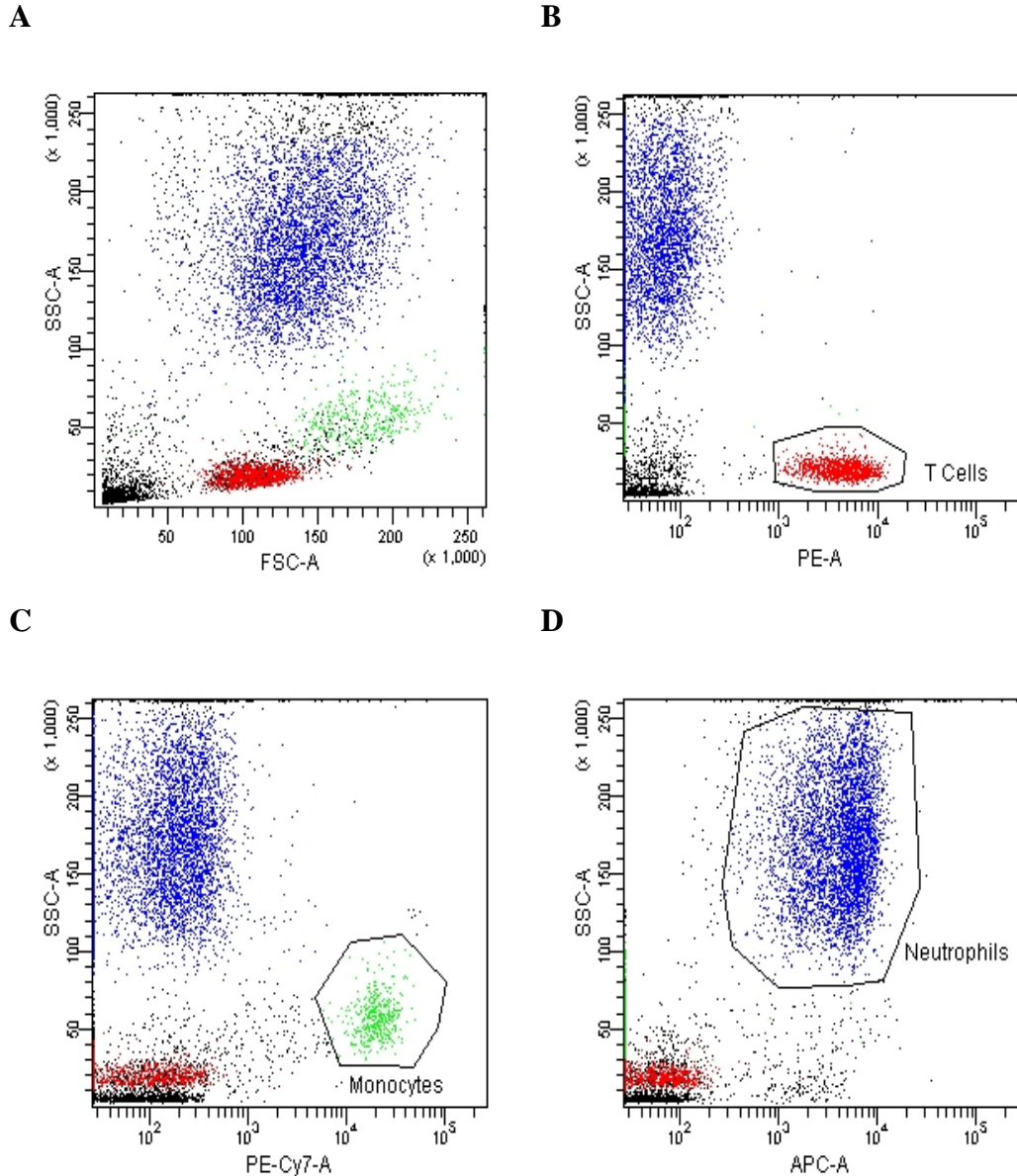


**Figure 3.4: Cell viability (A), caspase-3 (B) and necrosis (C) measurements of U937 cells in response to 0-6 hour heat shock treatment at 37.7°C–46.2°C and following 1 hour recovery at 37°C.**

Data are presented as SEM, n=3. Significance shown as difference from 37.7°C between mean time points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001).

### 3.3.2 Induction of apoptotic or necrotic cell death following treatment of human leukocyte cells with heat stress.

Human leukocytes taken from healthy volunteers' were isolated from whole blood and separated into different leukocyte subtypes using FSC/SSC and labelled antibodies to surface CD markers on the flow cytometer, to determine if there was any difference in their responses to heat stress and to measure the degree of apoptosis and necrosis between the different leukocyte subsets.



**Figure 3.5: Identification of leukocytes using antibodies to surface CD markers and FSC/SSC.**

Human leukocytes were gated using (A) FSC/SSC or antibodies to surface CD markers respectively (B) CD3 (■ Lymphocytes), (C) CD14 (■ Monocytes) or (D) CD15 (■ Neutrophils). Data are representative of three independent experiments and 10,000 events were recorded.

The induction of apoptosis and necrosis by different temperature treatments in human leukocytes are presented in Figures 3.6-3.10.

### **3.3.2.1 Induction of apoptosis in human leukocytes.**

Human leukocytes ( $3 \times 10^5$  cells/mL) were incubated at increasing temperatures of 37.7°C-44.4°C for up to four hours, followed by one hour recovery at 37°C. As shown in the dot blots presented in figure 3.6 and the time course experiments in figures 3.8-3.10, when leukocytes were exposed to temperatures above 37.7°C, a significant increase in apoptotic cell death ( $P<0.001$ ) was observed compared to control cells. A significant increase in the number of apoptotic neutrophils was observed at 42.7°C, approximately 10 % after a one hour incubation compared to control cells ( $P<0.001$ , Figure 3.8A-B). At temperatures lower than this (40.8°C) a non-significant increase was observed (Figure 3.8A-B), however at the increased temperature of 44.4°C after a one hour exposure, a significant increase in the number of apoptotic cells, approximately 20 % ( $P<0.001$ , Figure 3.8A-B) was achieved. Apoptosis was found to increase over time ( $P<0.001$ , Figure 3.8A-B), and a peak of apoptotic cell death was demonstrated at 42.7°C, approximately 90 % after a two hour ( $P<0.001$ , Figure 3.8A) and three hour ( $P<0.001$ , Figure 3.8B) exposure but declined with time. Apoptosis decreased by 50 % from 91 % ( $P<0.001$ ). Following incubation at 44.4°C there was also a significant decline ( $P<0.001$ ) in apoptosis in the neutrophil population from two hours (8 %,  $P<0.001$ ) up to four hours (12 %,  $P<0.05$ , Figure 3.8A-B).

Similarly as shown in figure 3.9, an increased temperature of 42.7°C after a one hour exposure led to a significant increase in the number of apoptotic lymphocytes, approximately 8 % compared to control cells ( $P<0.001$ , Figure 3.9A,  $P<0.05$ , Figure 3.9B). At the lower temperature of 40.8°C a non-significant increase was observed (Figure 3.8A-B), yet at the increased temperature of 44.4°C after a one hour exposure, a significant increase in the number of apoptotic cells, approximately 14 % ( $P<0.001$ , Figure 3.9A-B) resulted compared to control cells. Similar to the results observed with neutrophils, apoptosis increased over time and with increasing temperatures ( $P<0.001$ , Figure 3.9A-B). A peak in the number of apoptotic lymphocytes, approximately 85 % ( $P<0.001$ )

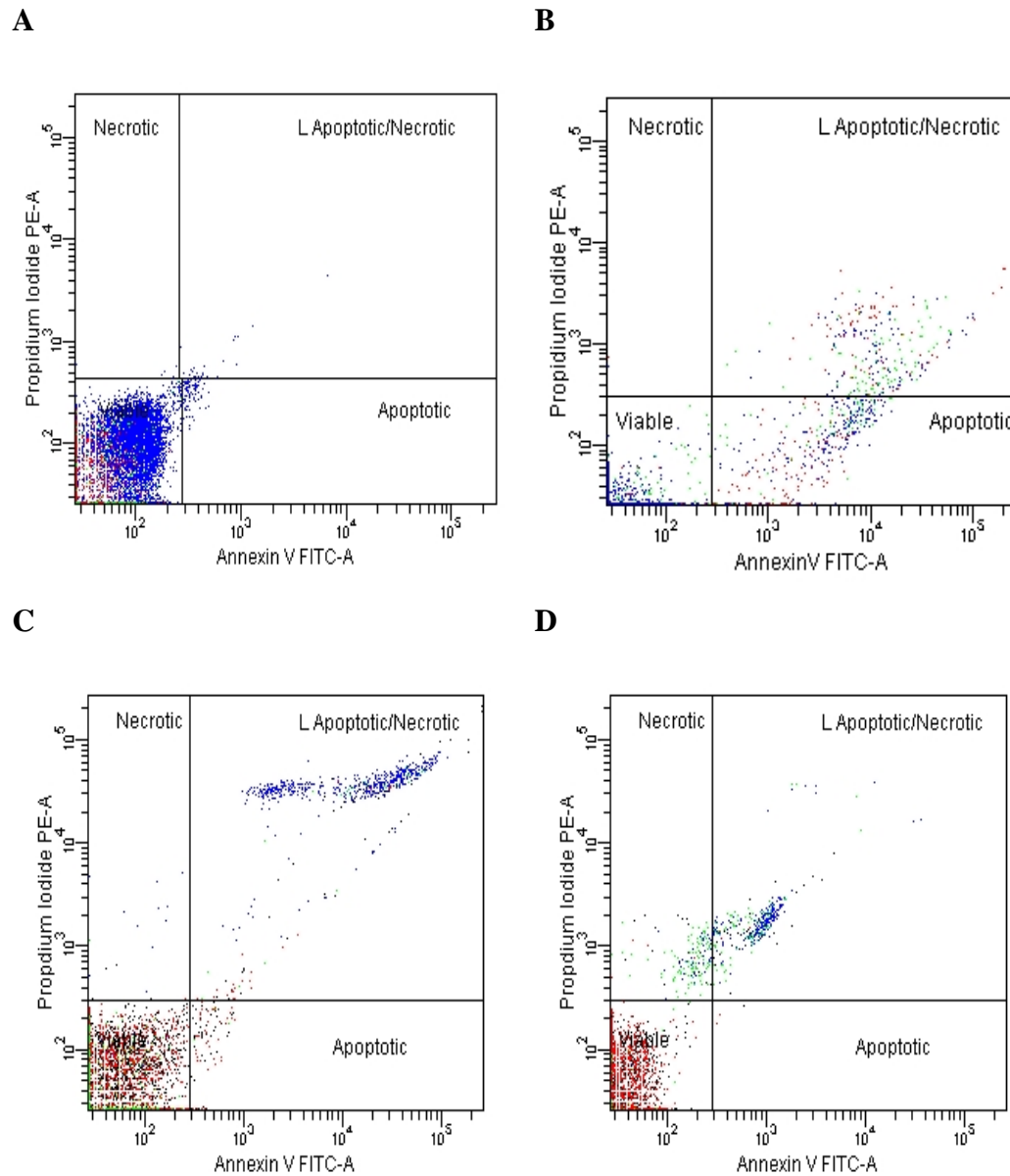
was achieved at 42.7°C after a three hour exposure and then declined to 60 % ( $P<0.001$ ) after a four hour exposure, a total decrease of 25 %.

In contrast, monocytes required a reduced temperature of 40.8°C in order to observe a significant increase in the number of apoptotic cells, approximately 35 % after a two hour incubation ( $P<0.001$ , Figure 3.10A-B) compared to control cells. At the lowest exposure time of one hour, a non-significant increase was observed (Figure 3.10A-B). Indeed, the peak temperature of apoptosis in monocytes was lower than both neutrophils and lymphocytes, 40.8°C which led to approximately 93 % apoptotic cell death ( $P<0.001$ , Figure 3.10A-B), although this was not observed until the longer length of exposure, three hours. After a four hour exposure to the elevated temperature of 40.8°C the number of apoptotic cells significantly reduced by 32 % from 93 % ( $P<0.05$ , Figure 3.10A-B). On the other hand, apoptosis increased with increasing temperatures (42.7°C and 44.4°C) over time ( $P<0.001$ , Figure 3.10A-B), and a significant increase in the number of apoptotic monocytes was observed after a one hour incubation compared to control cells ( $P<0.001$ , Figure 3.8 A-B). However, following incubation of monocytes at 44.4°C there was a significant decline ( $P<0.001$ ) in apoptosis from two hours (2-fold decrease) up to four hours (10-fold decrease,  $P<0.001$ , Figures 3.10A-B).

### **3.3.2.2 Induction of necrosis in human leukocytes.**

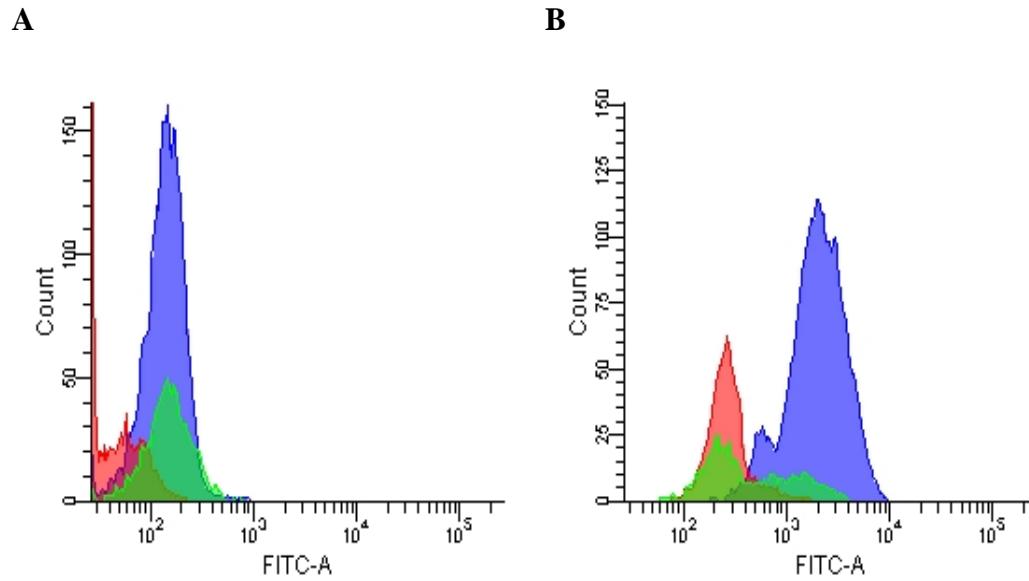
In addition to inducing apoptosis in human leukocytes, necrotic cell death was also induced in response to increasing temperatures over a period of four hours compared to control cells ( $P<0.001$ , Figures 3.8-3.10C). After a one hour incubation at 44.4°C, a significant increase in necrosis was observed in neutrophils, approximately 14 % ( $P<0.001$ , Figure 3.8C), in lymphocytes approximately 11 % ( $P<0.001$ , Figure 3.9C) and in monocytes approximately 12 % ( $P<0.001$ , Figure 3.10C) compared to control cells. At temperatures lower than this (40.8°C and 42.7°C) a non-significant increase was observed (Figures 3.8-3.10C). As the length of exposure to elevated temperatures increased, necrosis was also found to significantly increase compared to control cells ( $P<0.05$  to  $P<0.001$  to  $P<0.001$ , Figures 3.8-3.10C). After four hours of incubation all elevated temperatures resulted with a significant increase in necrotic cell death compared to control cells ( $P<0.05$  to  $P<0.001$ , Figures 3.8-

3.10C). Indeed, treatment at 44.4°C for four hours resulted with 92 % necrotic neutrophils ( $P<0.001$ ), 90 % necrotic lymphocytes ( $P<0.001$ ) and 98 % necrotic monocytes ( $P<0.001$ ) compared with the no treatment control.



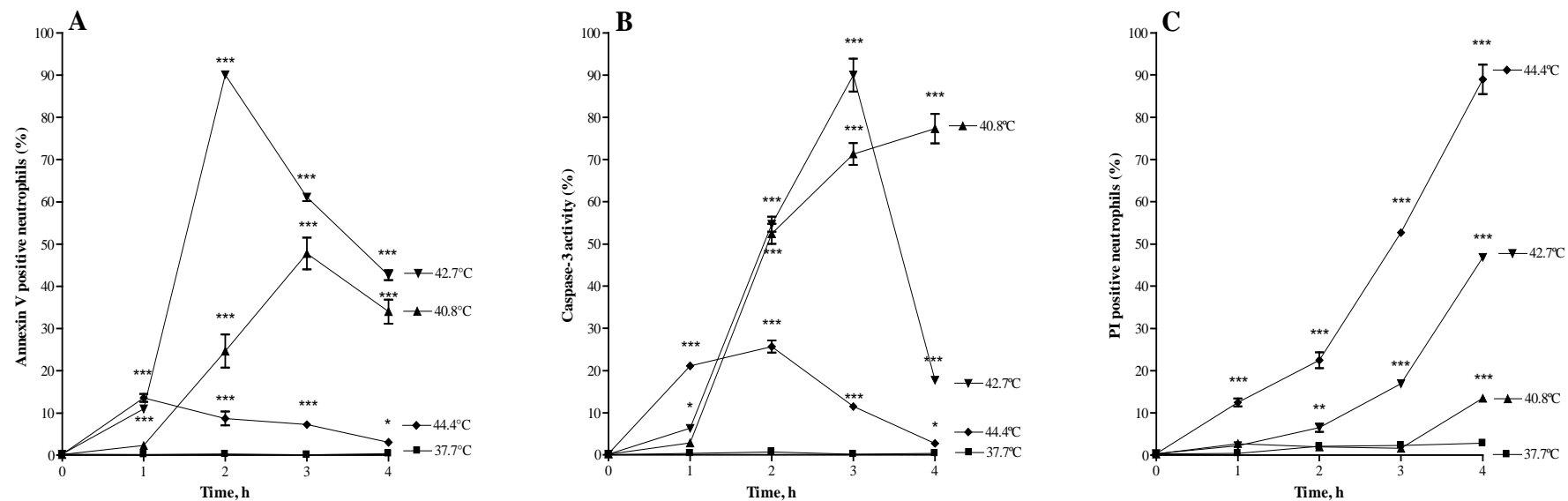
**Figure 3.6: Annexin V and PI assay performed on human leukocytes when incubated for 4 hours at 37.7°C-44.4°C and following 1 hour recovery at 37°C.**

Lymphocytes (■), monocytes (■) and neutrophils (■) were gated using CD3, CD14, CD15 and FSC/SCC respectively. (A) Control 37.7°C (4 hours) (B) Heat Shock 40.8°C (4 hours), (C) Heat Shock 42.7°C (4 hours) and (D) Heat Shock 44.4°C (4 hours). Data are representative of three independent experiments and 10,000 events were recorded.



**Figure 3.7: Caspase-3 analysis of human leukocytes was performed using flow cytometry.**

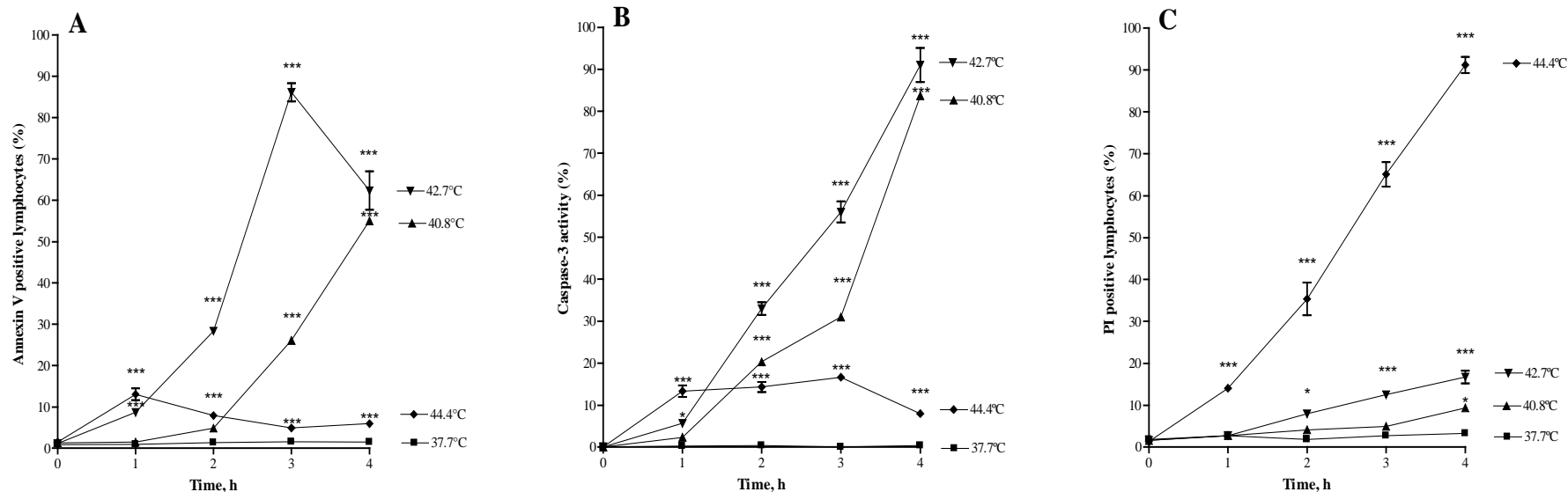
Human leukocytes were treated at 42.7°C for 1 hour and gated using FSC/SSC and antibodies to surface CD markers respectively, lymphocytes (CD3 ■), monocytes (CD14 ■) and neutrophils (CD15 ■). (A) No stain control sample. (B) Caspase-3 activity. Data are representative of three independent experiments and 10,000 events were recorded.



**Figure 3.8: Percentage of Annexin V (A), caspase-3 (B) and PI (C) positive neutrophils in response to 0-4 hour heat shock treatment at 37.7°C-44.4°C and following 1 hour recovery at 37°C.**

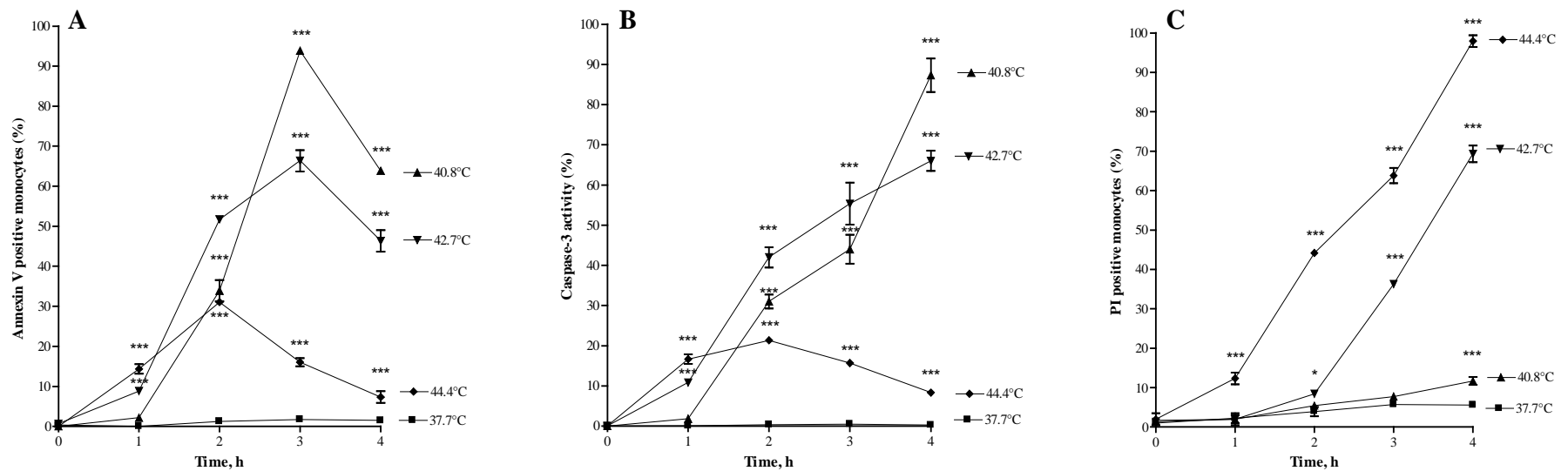
Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 37.7°C between mean time points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001).





**Figure 3.9: Percentage of Annexin V (A), caspase-3 (B) and PI (C) positive lymphocytes in response to 0-4 hour heat shock treatment at 37.7°C-44.4°C and following 1 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 37.7°C between mean time points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\*\* (P<0.001).



**Figure 3.10: Percentage of Annexin V (A), caspase-3 (B) and PI (C) positive monocytes in response to 0-4 hour heat shock treatment at 37.7°C-44.4°C and following 1 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM n=3. Significance shown as difference from 37.7°C between mean time points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\*\* (P<0.001).

### **3.3.3 Expression of surface and whole cell Hsp72 in untreated and heat stressed U937 cells.**

The expression of surface (membrane-bound in non-permeabilized cells) and whole cell (surface and intracellular in permeabilized cells) Hsp72 in U937 cells under control conditions (1 hour at 37°C) and after the heat treatment protocol (1 hour heat shock at 42°C, followed by 3 hour recovery at 37°C) was determined by flow cytometry (Figures 3.11-3.12) and fluorescence microscopy (Figures 3.13-3.14) respectively. The main objective of this part of the study was to examine the expression of Hsp72 under normal conditions and to investigate whether heat shock treatment leads to altered HSP surface and whole cell Hsp72 expression.

#### **3.3.3.1 Surface and whole cell expression of Hsp72 in U937 cells.**

The initial experiments shown in figures 3.11-3.12 reveal U937 cells express low base line levels of Hsp72, both on the cell surface, and on/inside U937 cells under control conditions. The results shown in figure 3.12 suggest the base line level of whole cell Hsp72 to be greater than cell surface Hsp72, however despite these differences the results were found to be non-significant (Figure 3.12). Consistent with the observations of flow cytometry, fluorescence microscopy (Figures 3.13-3.14) showed Hsp72 expressions both on the cell surface and on/inside U937 cells under control conditions using the mouse monoclonal anti-human Hsp72: FITC conjugated antibody coupled with DAPI labelling in order to detect the cells nuclei. The results demonstrated no co-localisation of Hsp72 (green) with the nucleus (blue) in control cells, indicating that the Hsp72 protein must be located in the cytosol.

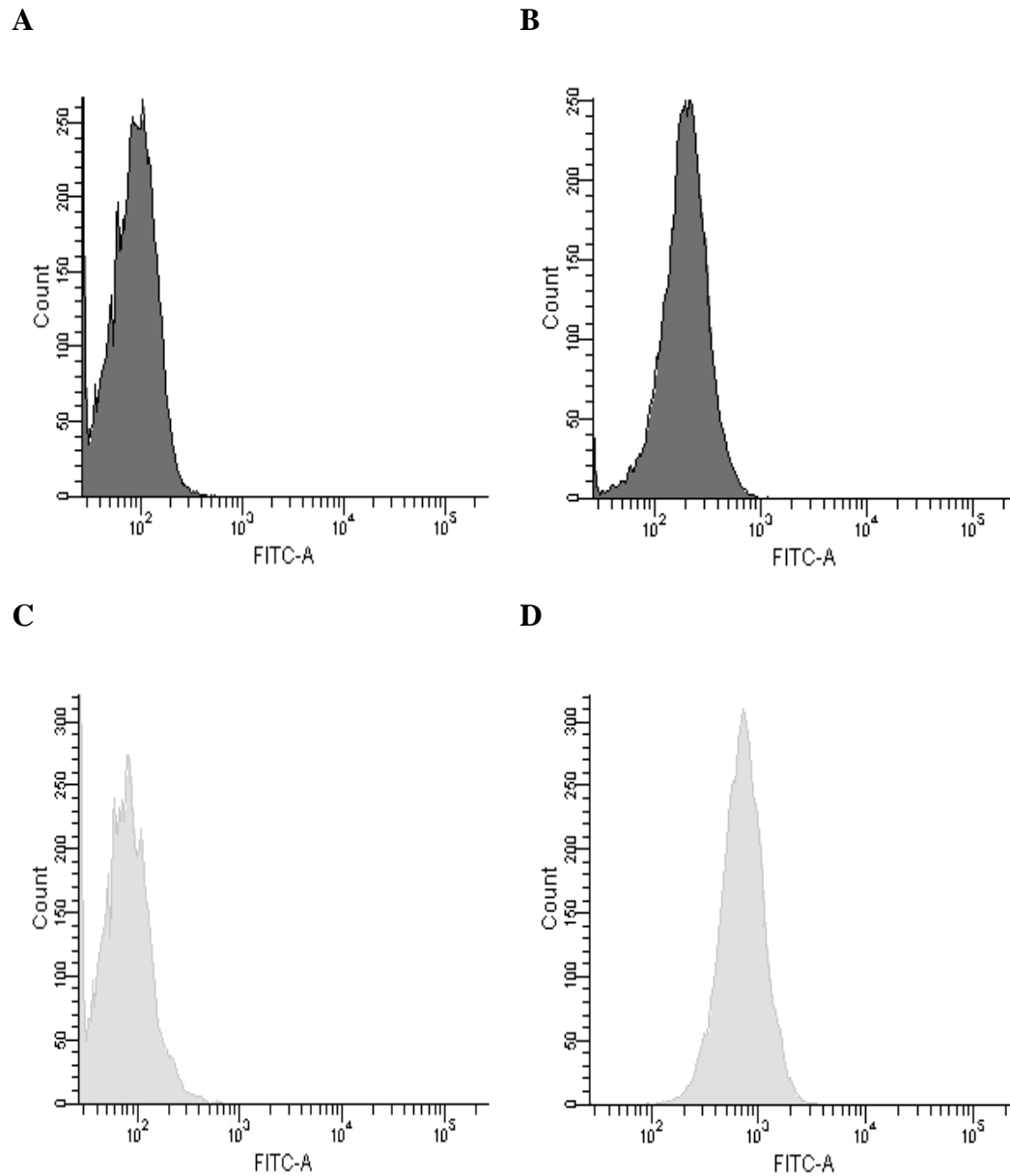
#### **3.3.3.2 Surface and whole cell expression of Hsp72 in heat stressed U937 cells.**

Following incubation of U937 cells at 42°C for one hour, an almost 13-fold increase ( $P < 0.001$ ) of cell surface Hsp72 was seen compared to untreated controls. In addition, an 11-fold increase ( $P < 0.001$ ) of whole cell Hsp72 was seen compared to untreated controls (Figure 3.12). Despite the difference in fold increase, the whole cell expression of Hsp72 was found to be significantly greater than cell surface Hsp72 ( $P < 0.01$ , Figure 3.12). These results indicate

following heat shock treatment, Hsp72 up-regulation and localisation is mainly concentrated within U937 cells (Figure 3.12). These results were confirmed by fluorescence microscopy (Figures 3.13-3.14), and revealed increased cell surface and whole cell Hsp72 expression after heat shock treatment at 42°C for one hour compared to untreated cells, but no co-localisation of Hsp72 with the nucleus was observed.

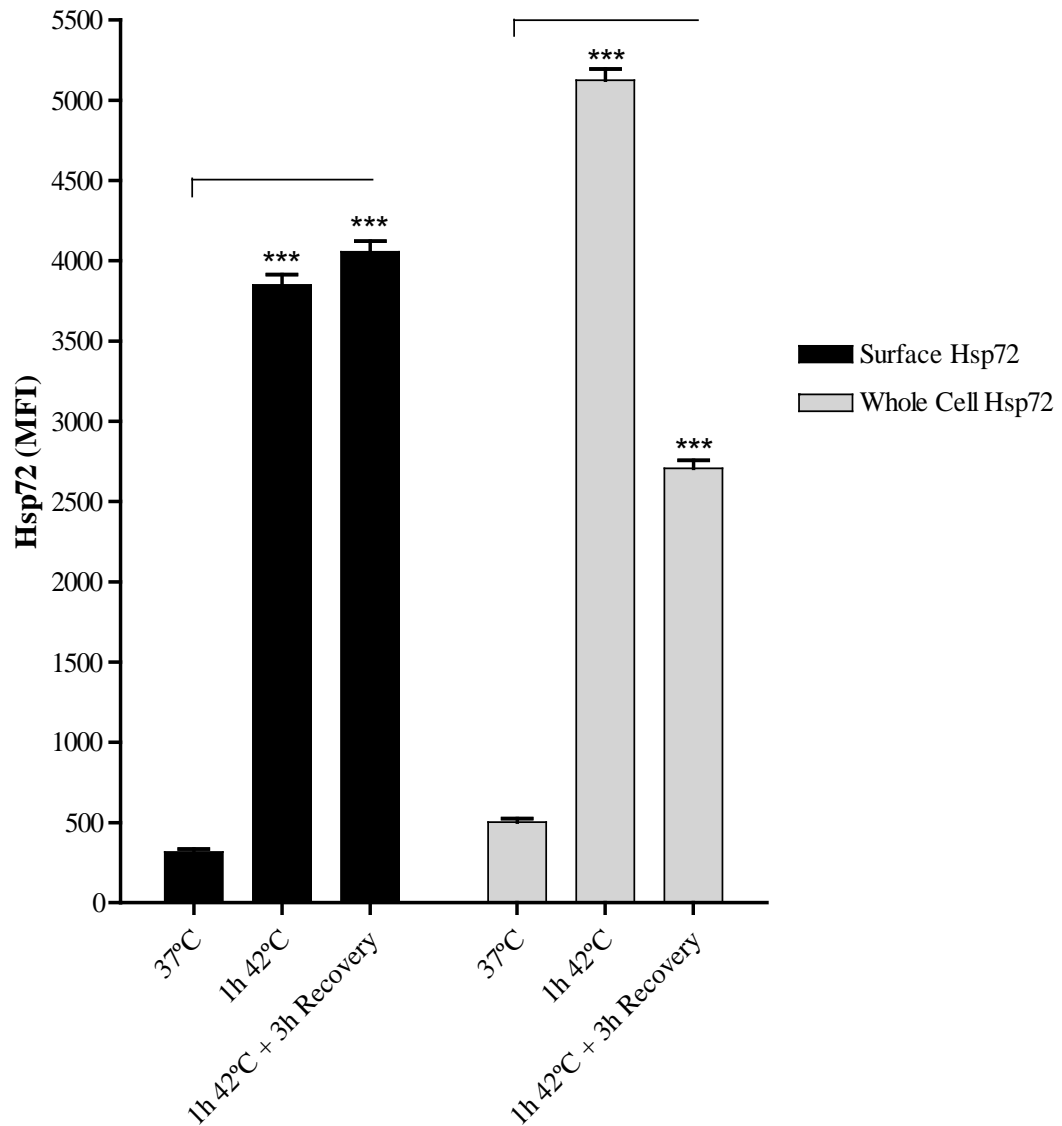
#### **3.3.3.3 Surface and whole cell expression of Hsp72 in heat stressed U937 cells following a three hour recovery at 37°C.**

In response to a three hour recovery at 37°C, an almost 1-fold increase ( $P < 0.001$ ) of cell surface Hsp72 was seen compared to the 42°C heat shocked sample. In contrast, a 1-fold decrease ( $P < 0.05$ ) of whole cell Hsp72 was seen compared to the 42°C heat shocked sample, however levels did not decrease to equal or below the value of the untreated control (Figure 3.12). The results obtained by flow cytometry were again confirmed by fluorescence microscopy (Figures 3.13-3.14), and revealed heat shock significantly enhanced Hsp72 expression, however no evidence for co-localisation of Hsp72 within the nucleus was seen, suggesting Hsp72 must be located within the cytosol.



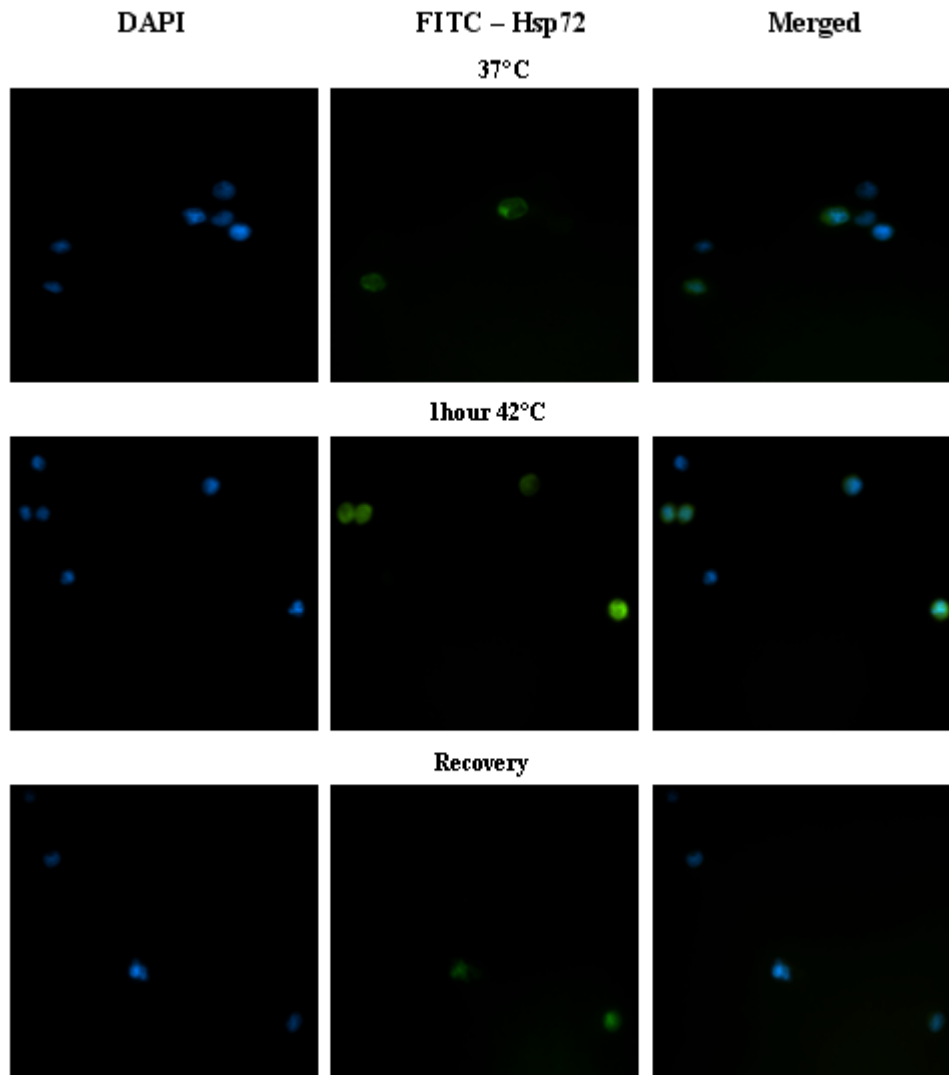
**Figure 3.11: Surface (A and B) and whole cell (C and D) Hsp72 analysis of U937 cells was performed using flow cytometry.**

U937 cells were treated at 37°C for 1 hour and gated using FSC/SSC. (A, B) No stain control samples. (B, D) Hsp72 expression. Data are representative of three independent experiments and 10,000 events were recorded.

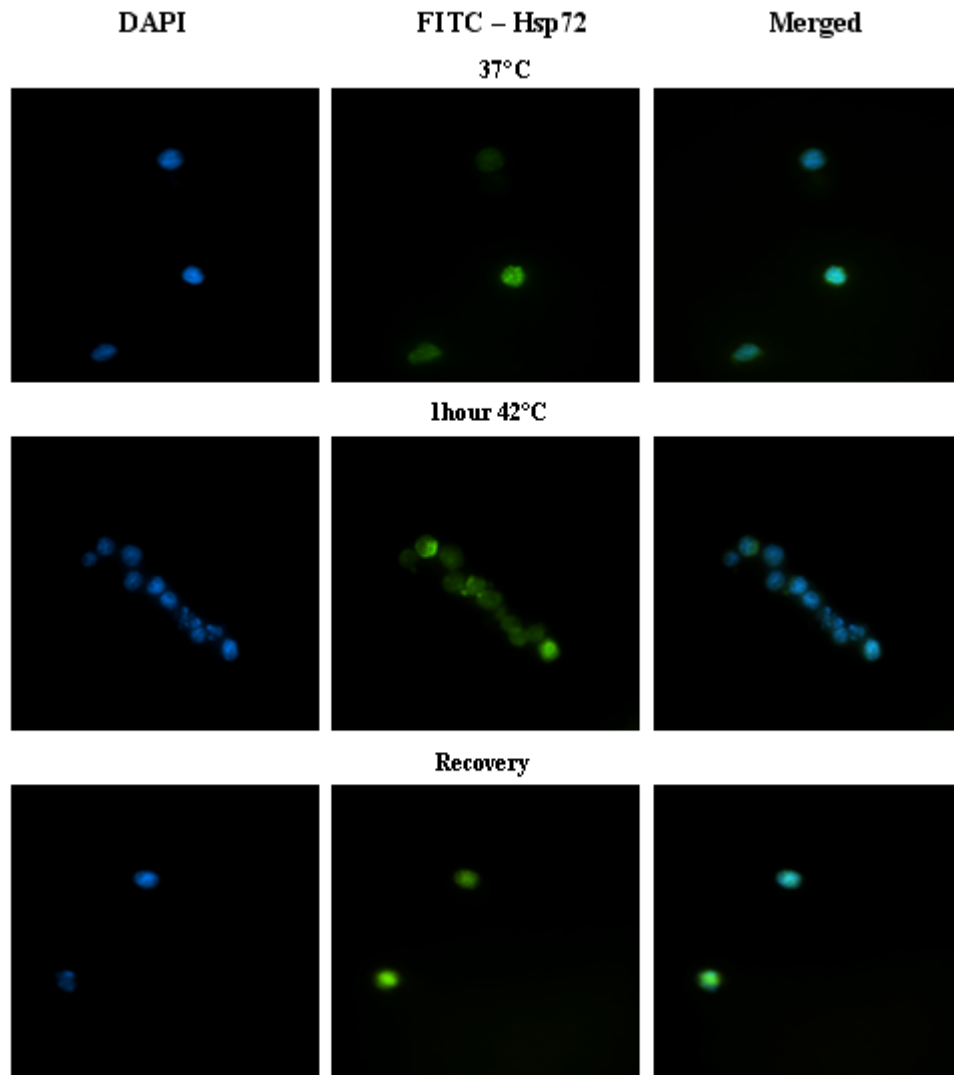


**Figure 3.12: Expression of Hsp72 in U937 cells at 37°C, in response to 1 hour 42°C heat shock treatment and following 3 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from 37°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



**Figure 3.13: Hsp72 surface expression on U937 cells at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.** Images were acquired at a magnification of x 40 set up in DAPI (nuclear stain) or FITC (Hsp72 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



**Figure 3.14: Whole cell Hsp72 expression in U937 cells at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Images were acquired at a magnification of x 40 set up in DAPI (nuclear stain) or FITC (Hsp72 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



### **3.3.4 Expression of surface and whole cell Hsp27 in untreated and heat stressed human leukocytes.**

To characterise the stress response in human leukocytes, the cell surface and whole cell expression of the small stress protein Hsp27 was measured in control and heat shocked cells. The main objective of this part of the study was to investigate Hsp27 expression in human leukocytes under control conditions and to determine whether elevated temperature stress leads to altered HSP expression on the cell surface and on/inside human leukocytes.

The expression of cell surface and whole cell Hsp27 on human leukocytes are presented in Figures 3.15-3.20.

#### **3.3.4.1 Surface and whole cell expression of Hsp27 in human leukocytes.**

The experiments shown in figures 3.16-3.18 reveal human leukocytes to express low base line levels of Hsp27, on the cell surface (Figure 3.16) and on/inside (Figure 3.18) human leukocytes under control conditions. The results suggest human leukocytes express increased levels of whole cell Hsp27 compared to cell surface levels, however only neutrophils and monocytes revealed significant differences ( $P < 0.001$ , Figures 3.16-3.18), the levels for lymphocytes were found to be non-significant (Figures 3.16-3.18). Between the leukocyte subtypes, neutrophils were found to express increased levels of both cell surface and whole cell Hsp27 compared to lymphocytes ( $P < 0.001$ , Figures 3.16-3.18) and monocytes ( $P < 0.05$ , Figures 3.16-3.18). These results suggest Hsp27 is expressed in untreated human leukocytes but its expression varies between the different leukocyte subtypes. These results were confirmed by fluorescence microscopy (Figures 3.17-3.19). The Western blot of human leukocyte membrane extracts and cell lysate probed with a specific antibody for Hsp27 (Figure 3.20), showed a banding at approximately 27 kDa in the untreated cell lysate sample (Lanes 5), however no band was seen in the untreated membrane extract (Lane 1), thus confirming the localisation of Hsp27 inside the cell but not with the cell surface.

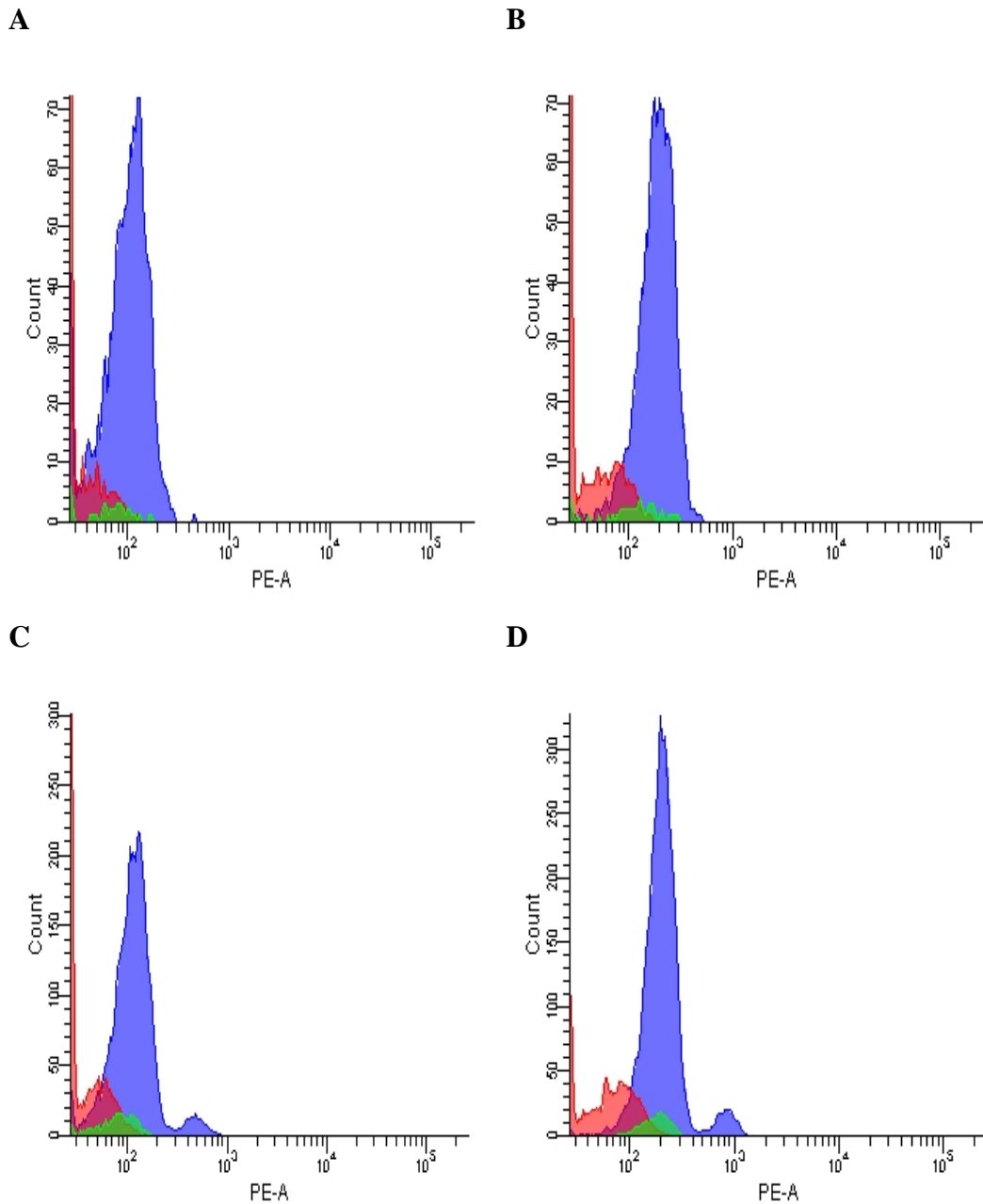
#### **3.3.4.2 Surface and whole cell expression of Hsp27 in heat stressed human leukocytes.**

When human leukocytes were stressed by heat at 42°C for one hour, a rapid increase in Hsp27 expression occurred resulting with a significant up-regulation of both cell surface (Figure 3.16) and whole cell (Figure 3.18) Hsp27 compared to untreated cells. However, the expression varied amongst the leukocyte subtypes. A 7-fold increase ( $P<0.01$ ) of cell surface Hsp27 was seen compared to untreated controls in neutrophils (Figure 3.16), in addition to an almost 5-fold increase ( $P<0.001$ ) of whole cell Hsp27 (Figure 3.18). Despite the difference in fold increase, these results found following heat shock treatment, Hsp27 up-regulation and localisation is mainly concentrated inside neutrophils (Figure 3.18). On the other hand, Hsp27 expression in lymphocytes did not show any significant difference between cell surface and whole cell Hsp27. In both cases an almost 16-fold increase was observed ( $P<0.001$ , Figures 3.16-3.18), whilst monocytes appeared to exhibit the highest induction of Hsp27 expression following heat stress. An almost 42-fold increase ( $P<0.001$ ) of cell surface Hsp27 was seen compared to untreated cells (Figure 3.16), in addition to a 40-fold increase ( $P<0.001$ ) of whole cell Hsp27 (Figure 3.18). These results suggest following heat shock treatment, Hsp27 up-regulation and localisation is mainly concentrated on the cell surface of monocytes (Figures 3.16). These results were confirmed by fluorescence microscopy (Figures 3.17-3.19), and revealed increased cell surface and whole cell Hsp27 expression after heat shock treatment compared to untreated cells, but no co-localisation of Hsp27 with the nucleus was observed. In addition, the results of Western blotting (Figure 3.20) showed increased band intensity at approximately 27 kDa in the samples containing leukocyte membrane extracts and cell lysate heat shocked for one hour at 42°C compared to the untreated samples (Lanes 2 and 6).

#### **3.3.4.3 Surface and whole cell expression of Hsp27 in heat stressed human leukocytes following a three hour recovery at 37°C.**

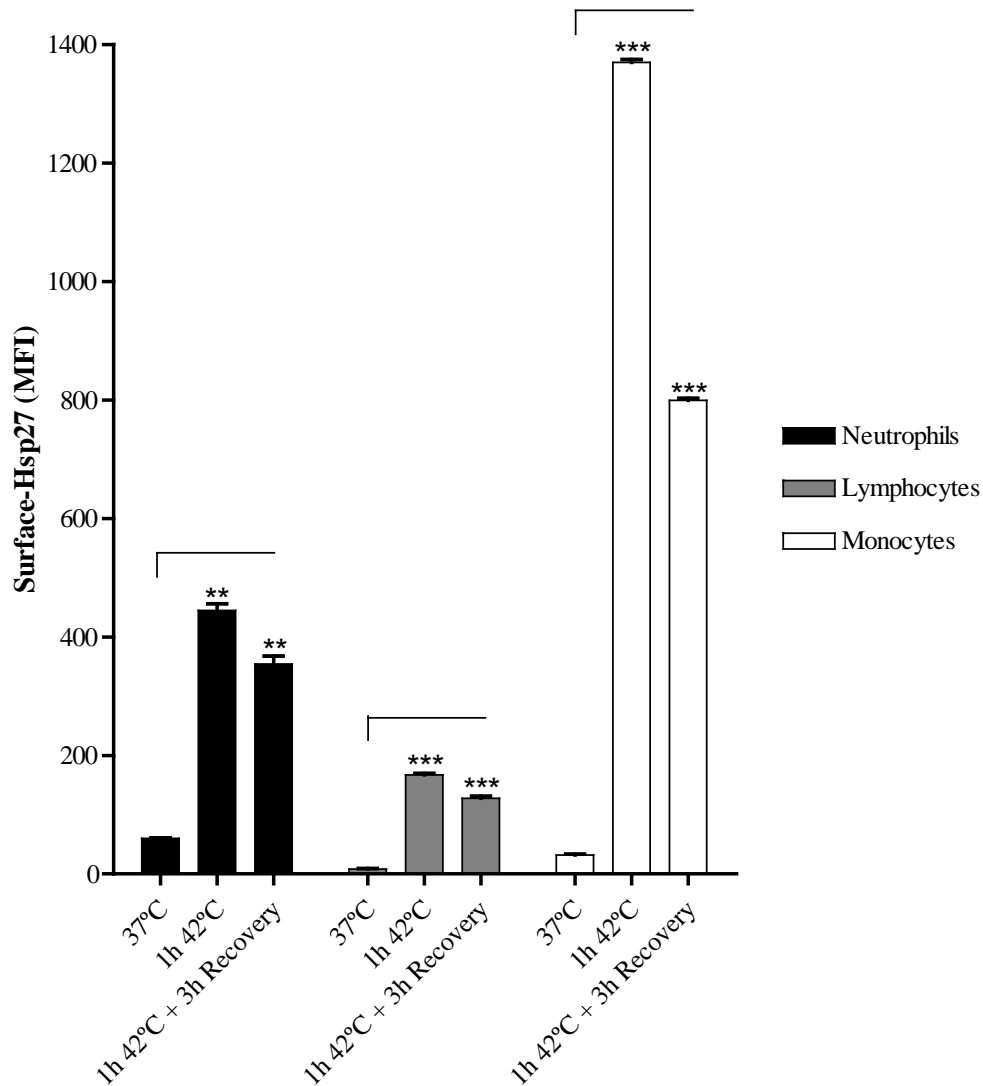
In response to a three hour recovery at 37°C, the expression of whole cell Hsp27 was slightly decreased in all leukocyte subtypes, however levels were not reduced to values that were equal or below base line levels (Figure 3.18). Indeed, a 1-fold decrease ( $P<0.05$ ) of whole cell Hsp27 was seen in neutrophils

compared to the 42°C heat shocked sample (Figure 3.18). Similarly, a 1-fold decrease of whole cell Hsp27 was seen in lymphocytes compared to the 42°C heat shocked sample, however the difference was found to be non-significant (Figure 3.18). Furthermore, a 5-fold decrease ( $P < 0.001$ ) of whole cell Hsp27 was seen in monocytes compared to the 42°C heat shocked sample (Figure 3.18). The decrease of whole cell Hsp27 corresponded with a significant down-regulation of cell surface Hsp27 compared to untreated cells (Figure 3.16), indicating the potential translocation of Hsp27 in human leukocytes. A 1-fold decrease ( $P < 0.01$ ) of cell surface Hsp27 was seen compared to the 42°C heat shocked neutrophils, in addition to a 1-fold decrease ( $P < 0.001$ ) of cell surface Hsp27 in lymphocytes, and finally an almost 2-fold decrease ( $P < 0.001$ ) of cell surface Hsp27 was seen in monocytes compared to the 42°C heat shocked samples (Figure 3.16). These results suggest the movement of Hsp27 within cells and its potential translocation to the cell surface and release. The results obtained by flow cytometry were confirmed by fluorescence microscopy (Figures 3.17-3.19), and revealed heat shock significantly enhanced Hsp27 expression and potentiated its translocation, however no evidence for co-localisation of Hsp27 within the nucleus was seen, suggesting Hsp27 must be located within the cytosol. Western blotting (Figure 3.20) showed evidence of decreased band intensity at approximately 27 kDa in the samples containing leukocyte membrane extracts and cell lysate that were heat shocked for one hour at 42°C followed by recovery for three hours at 37°C compared to the samples that were heat shocked for one hour at 42°C (Lanes 3 and 7).



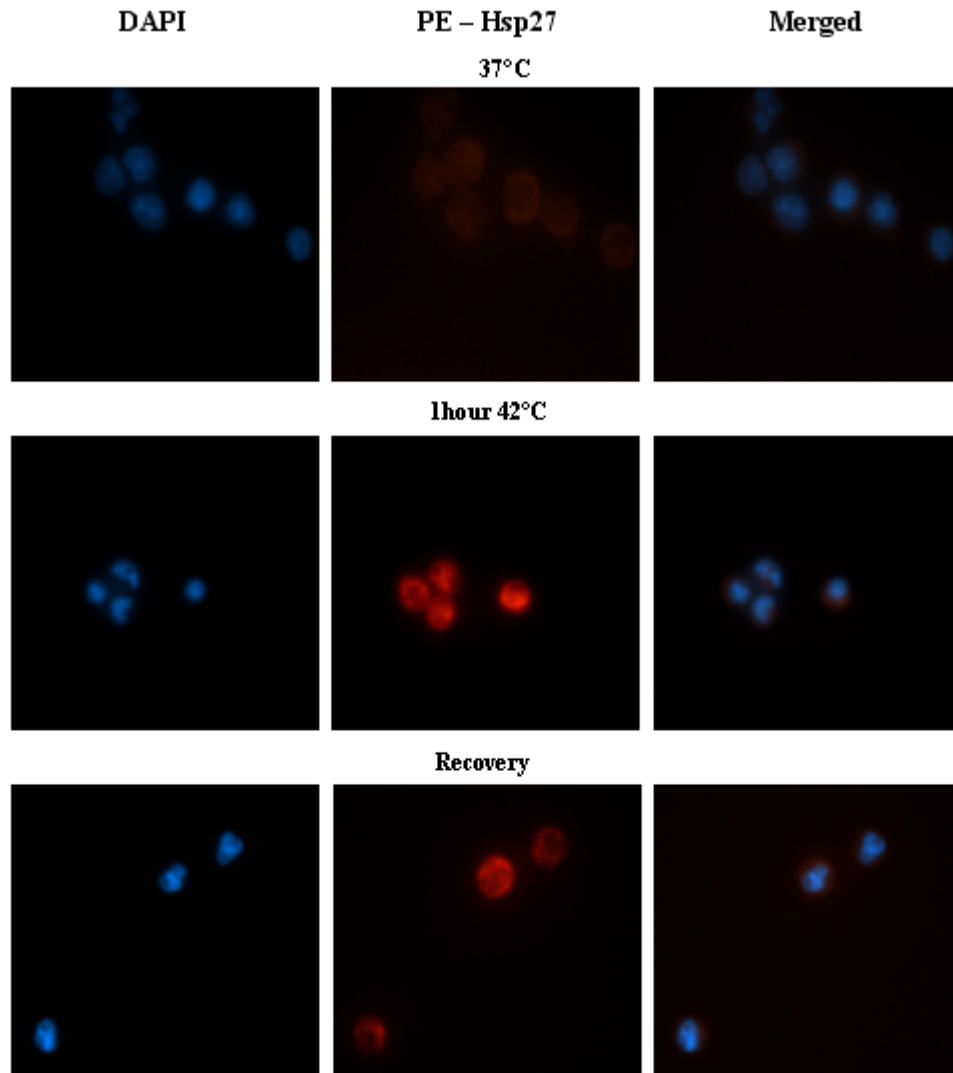
**Figure 3.15: Surface (A and B) and whole cell (C and D) Hsp27 analysis of human leukocytes was performed using flow cytometry.**

Human leukocytes were treated at 37°C for 1 hour. Neutrophils (■), lymphocytes (■) and monocytes (■) were gated using CD3, CD14, CD15 and FSC/SSC respectively. (A, C) No stain control samples. (B, D) Hsp27 expression. Data are representative of three independent experiments and 10,000 events were recorded.



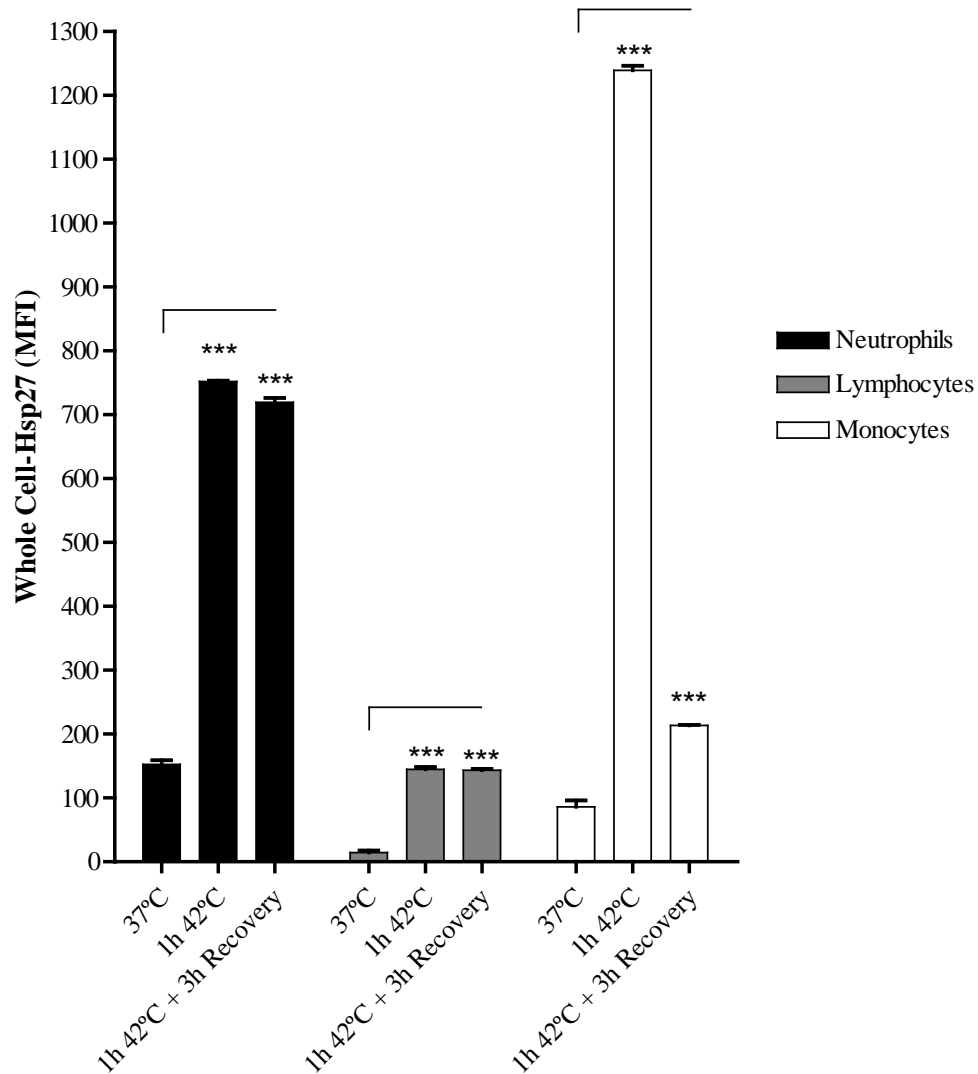
**Figure 3.16: Surface expression of Hsp27 on leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 37°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* (P<0.001), \*\*\* (P<0.001).



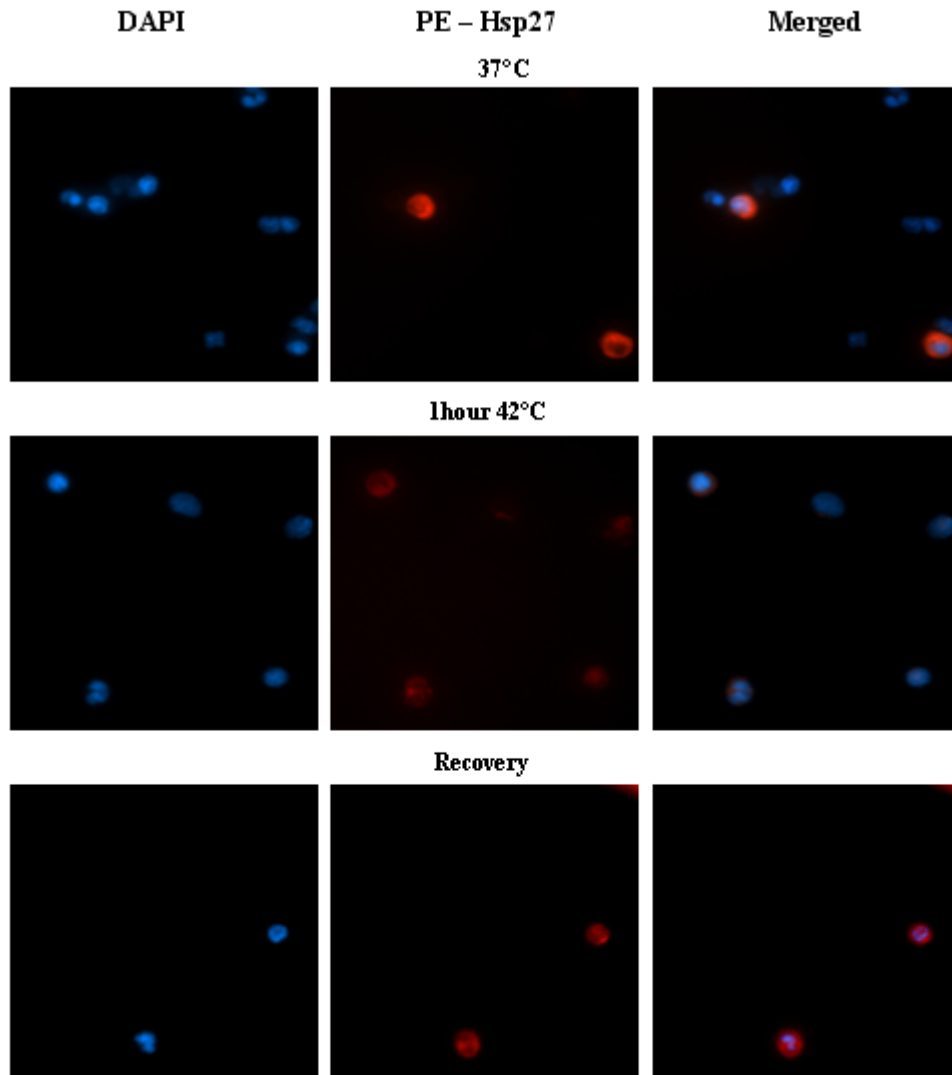
**Figure 3.17: Surface expression of Hsp27 on leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Images were acquired at a magnification of x 60 set up in DAPI (nuclear stain) or TRITC (Hsp27 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



**Figure 3.18: Whole cell Hsp27 expression in leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 37°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).



**Figure 3.19: Whole cell Hsp27 expression in leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Images were acquired at a magnification of x 60 set up in DAPI (nuclear stain) TRITC (Hsp27 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.





**Figure 3.20: Western blot of membrane associated and whole cell Hsp27 protein from human leukocytes.**

A 10 % gel was cast and loaded with the following under reduced conditions: Lane 1 - leukocyte membrane extract at 37°C; Lane 2 - leukocyte membrane extract heat shocked for 1 hour at 42°C; Lane 3 - leukocyte membrane extract heat shocked and following 3 hours of recovery at 37°C; Lane 4 - pure human Hsp27 (1 µg/mL); Lane 5 - cell lysate at 37°C; Lane 6 - cell lysate heat shocked for 1 hour at 42°C; Lane 7 - cell lysate heat shocked and following 3 hours of recovery at 37°C and Lane 8 - pure human Hsp27 (1 µg/mL). The blot was probed with 1:10,000 dilution of biotinylated anti-Hsp27 followed by avidin at 1:50,000 dilution, it was stained with SuperSignal® west pico chemiluminescent substrate and exposed for 480 seconds.

### **3.3.5 Expression of surface and whole cell Hsp60 in untreated and heat stressed human leukocytes.**

Human leukocytes from both control and heat shocked samples were analysed for the expression of cell surface and whole cell Hsp60 determined quantitatively by flow cytometry (Figures 3.22-3.25), fluorescence microscopy (Figures 3.24-3.26) and Western blotting (Figure 3.27) respectively. The main objective of this part of the study was to investigate the expression of Hsp60 under control conditions and to determine whether cellular stress leads to altered HSP expression.

The expression of cell surface and whole cell Hsp60 on human leukocytes are presented in Figures 3.21-3.26.

#### **3.3.5.1 Surface and whole cell expression of Hsp60 in human leukocytes.**

The experiments shown in figures 3.22-3.24 suggest human leukocytes to express low base line levels of Hsp60, on the cell surface (Figure 3.22) and on/inside human leukocytes (Figure 3.24) under control conditions. The results reveal the base line level of whole cell Hsp60 to be greater than cell surface Hsp60, however despite these differences only monocytes resulted in a significant difference ( $P < 0.01$ , Figures 3.22-3.24). The results for neutrophils and lymphocytes were both found to be non-significant (Figure 3.22-3.24). Between the leukocyte subtypes, neutrophils were found to express increased levels of both cell surface and whole cell Hsp60 compared to lymphocytes ( $P < 0.001$ , Figure 3.22,  $P < 0.05$ , Figure 3.24) and monocytes ( $P < 0.001$ , Figures 3.22-3.24). These results indicate Hsp60 is expressed in human leukocytes but its expression varies between the different leukocyte subtypes. Consistent with these observations, fluorescence microscopy (Figures 3.23-3.25) showed Hsp60 expressions both on the cell surface and on/inside human leukocytes under control conditions using the mouse monoclonal anti-human Hsp60: PE conjugated antibody coupled with DAPI labelling. The results demonstrated no co-localisation of Hsp60 (red) with the nucleus (blue) in control cells. Similarly, the Western blot of human leukocyte membrane extracts and cell lysate probed with a specific antibody for Hsp60 (Figure 3.26), showed a band at approximately 60 kDa in the untreated cell lysate sample (Lanes 5), however no

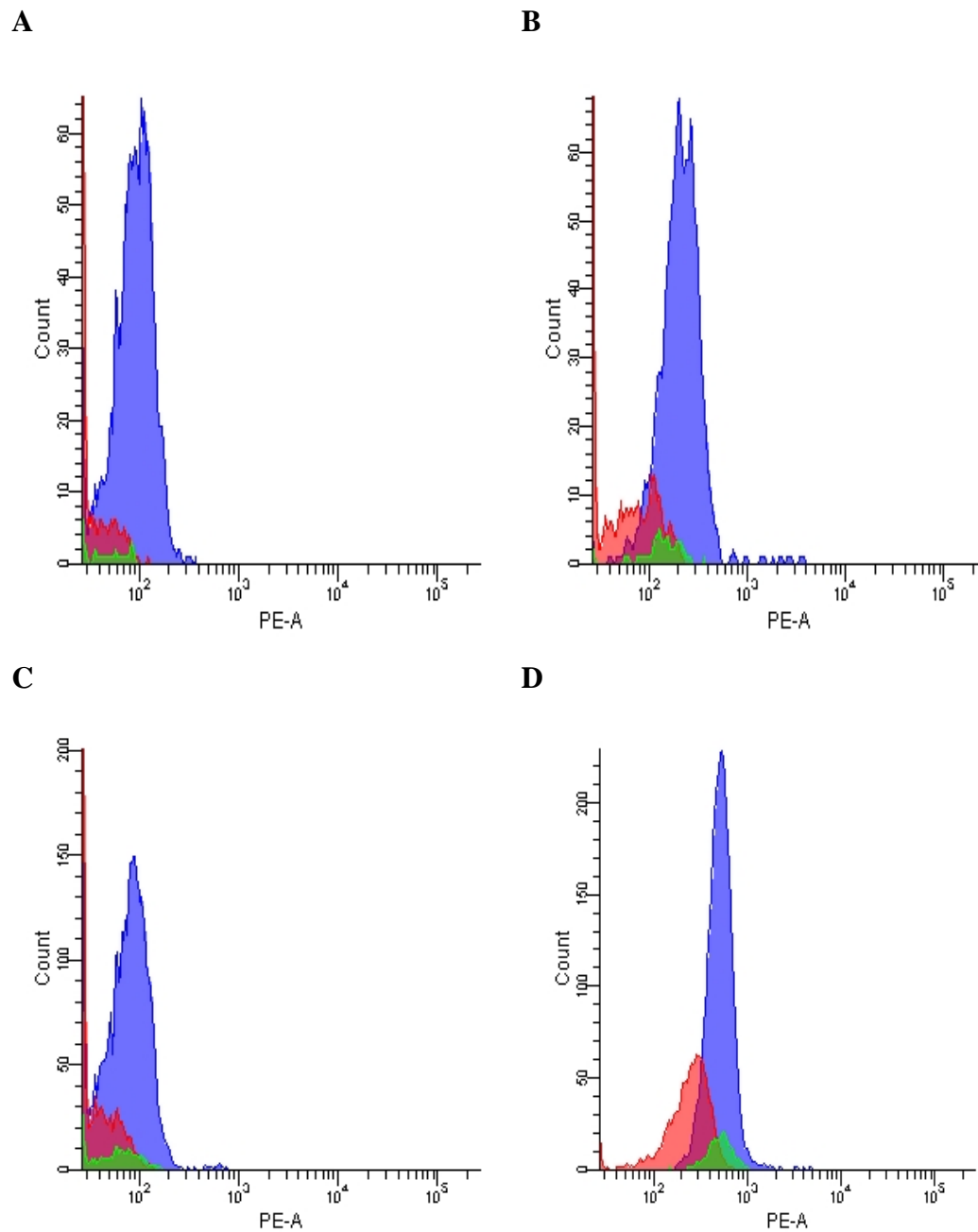
band was seen in the untreated membrane extract (Lane 1), confirming the localisation of Hsp60 inside the cell but not with the cell surface.

### **3.3.5.2 Surface and whole cell expression of Hsp60 in heat stressed human leukocytes.**

Following the incubation of human leukocytes at 42°C for one hour, a significant up-regulation of cell surface ( $P<0.001$ , Figure 3.22) and whole cell ( $P<0.001$ , Figure 3.24) Hsp60 expression resulted compared to untreated cells. However, the expression was found to vary between the leukocytes subtypes. An almost 5-fold increase ( $P<0.001$ ) of cell surface Hsp60 was seen compared to the untreated control in neutrophils (Figure 3.22), in addition to an almost 13-fold increase ( $P<0.001$ ) of whole cell expressed Hsp60 (Figure 3.24). In contrast, in monocyte cells a 26-fold increase ( $P<0.001$ ) of cell surface Hsp60 was seen compared to the untreated control (Figure 3.22), in addition to an almost 9-fold increase ( $P<0.001$ ) of whole cell Hsp60 (Figure 3.24). Despite the difference in fold increase, the up-regulation of Hsp60 in neutrophils was found to be significantly greater than monocytes ( $P<0.001$ , Figures 3.22-3.24). These results suggest following heat shock treatment, Hsp60 up-regulation and localisation is mainly concentrated within neutrophils and monocytes (Figure 3.22). In comparison, lymphocytes exhibited a reduced up-regulation of cell surface Hsp60, an 8-fold increase ( $P<0.001$ , Figure 3.22), and a 14-fold increase of whole cell Hsp60 ( $P<0.01$ , Figure 3.24) compared to the untreated control. However, the expression did not vary between cell surface and whole cell location, although a significant increase of Hsp60 expression compared to the untreated control was observed ( $P<0.001$ , Figures 3.22-3.24). These results were confirmed by fluorescence microscopy (Figures 3.23-3.25), and revealed increased cell surface and whole cell Hsp60 expression after heat shock treatment compared to untreated cells, but no co-localisation of Hsp60 with the nucleus was observed. In addition, the results of Western blotting (Figure 3.26) also showed increased band intensity at approximately 60 kDa in the samples containing human leukocyte cell lysate that was heat shocked for one hour at 42°C compared to untreated controls (Lanes 6), again no band was seen in the heat shocked membrane extract (Lane 2), confirming the localisation of Hsp60 within the cell but not with the cell surface.

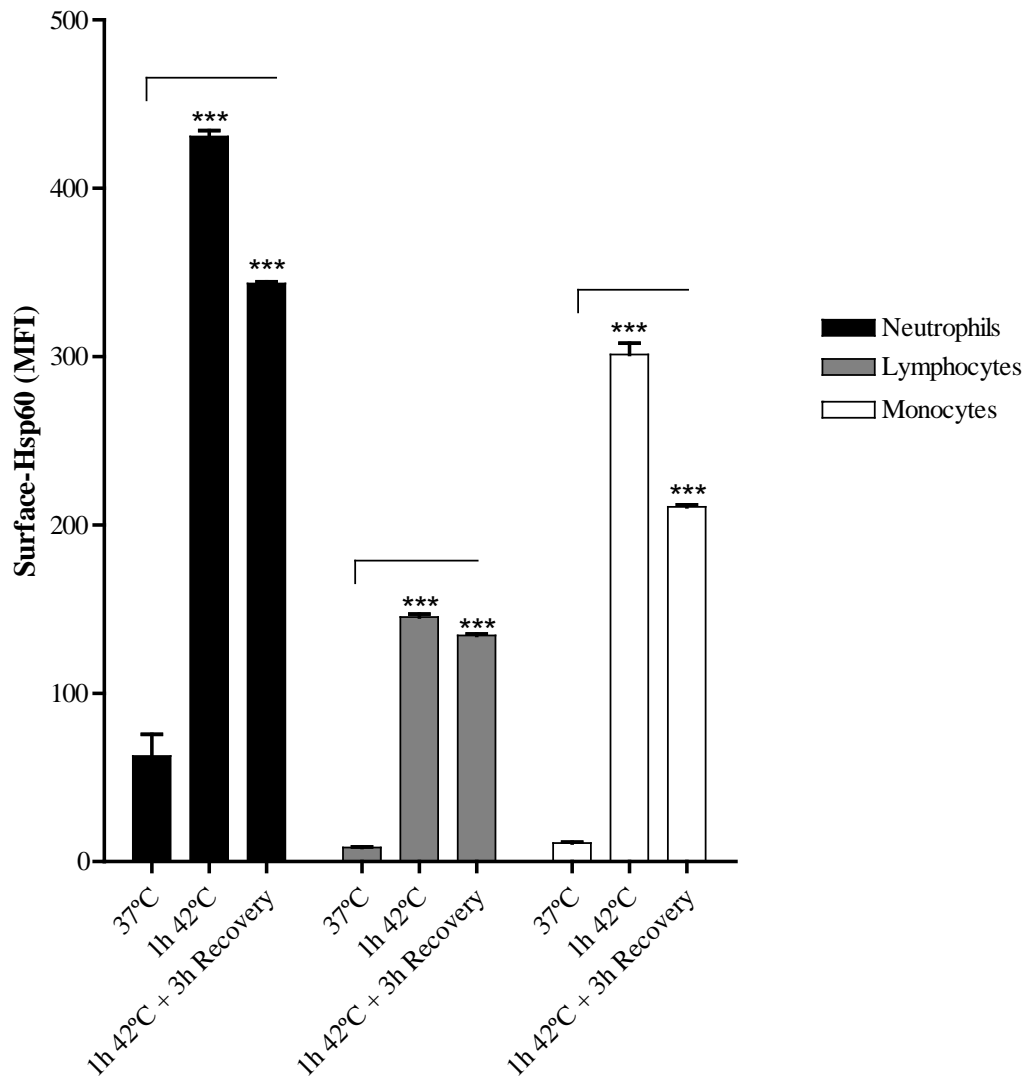
**3.3.5.3 Surface and whole cell expression of Hsp60 in heat stressed human leukocytes following a three hour recovery at 37°C.**

In response to a three hour recovery at 37°C, an almost 1-fold decrease ( $P<0.001$ ) of cell surface Hsp60 was seen in neutrophils compared to the 42°C heat shocked sample (Figure 3.22). Similarly, a 1-fold decrease of cell surface Hsp60 was also seen in both lymphocytes ( $P<0.01$ ) and monocytes ( $P<0.001$ ) compared to the 42°C heat shocked sample (Figure 3.22). However, levels were still increased compared to base line levels (Figure 3.22). In contrast to Hsp27, the decrease of cell surface Hsp60 corresponded with a significant up-regulation of whole cell Hsp60 ( $P<0.001$ , Figure 3.24). An almost 2-fold increase ( $P<0.001$ ) of whole cell Hsp60 was seen compared to the 42°C heat shocked sample in all leukocyte subtypes (Figure 3.24). These results suggest the movement of Hsp60 within cells, its potential translocation to the cell surface and also the induction of whole cell levels of Hsp60. The results obtained by flow cytometry were confirmed by fluorescence microscopy (Figures 3.23-3.25), and revealed heat shock significantly enhanced Hsp60 expression and potentiated its translocation, however no evidence for co-localisation of Hsp60 with the nucleus was obtained, suggesting Hsp60 must be located in the cytosol. Western blotting (Figure 3.26) showed evidence of increased band intensity at approximately 60 kDa in the sample containing human leukocyte cell lysate heat shocked plus recovery for three hours at 37°C compared to the samples that were heat shocked for one hour at 42°C (Lane 7), similarly no evidence of band intensity was seen in the membrane extract sample (Lane 3), confirming the presence of Hsp60 inside the cell but not with the cell surface.



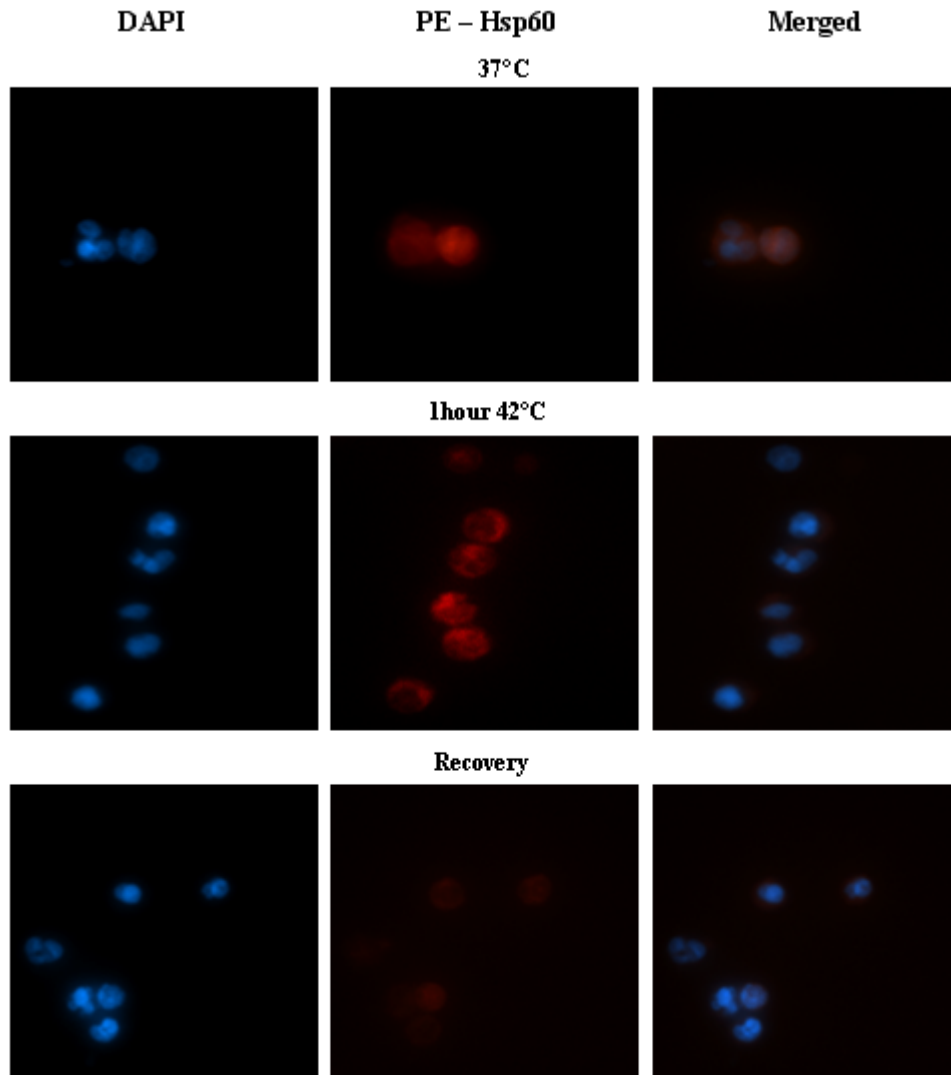
**Figure 3.21: Surface (A and B) and whole cell (C and D) Hsp60 analysis of human leukocytes was performed using flow cytometry.**

Human leukocytes were treated at 37°C for 1 hour. Neutrophils (■), lymphocytes (■) and monocytes (■) were gated using CD3, CD14 and CD15 and FSC/SSC respectively. (A, C) No stain control samples. (B, D) Hsp60 expression. Data are representative of three independent experiments and 10,000 events were recorded.



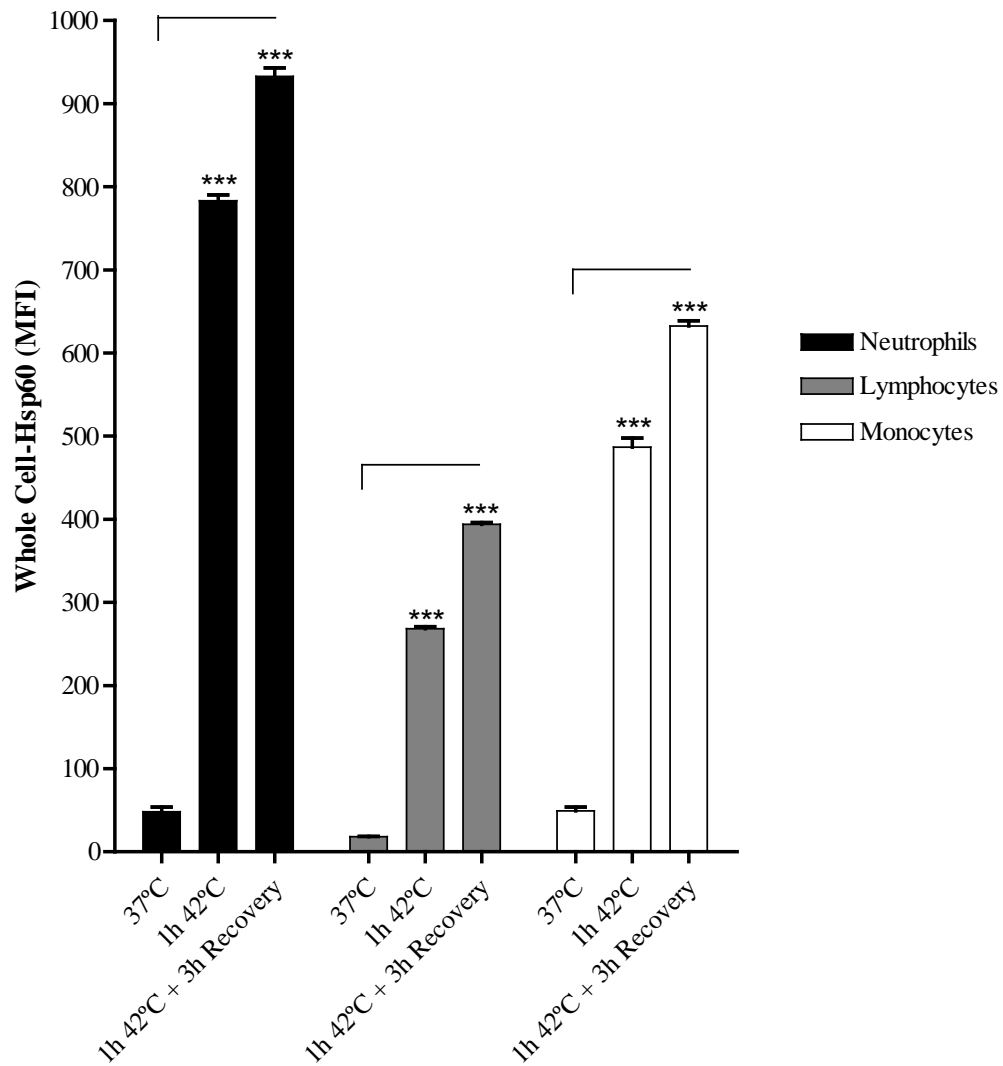
**Figure 3.22: Surface expression of Hsp60 on leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 37°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).



**Figure 3.23: Surface expression of Hsp60 in leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

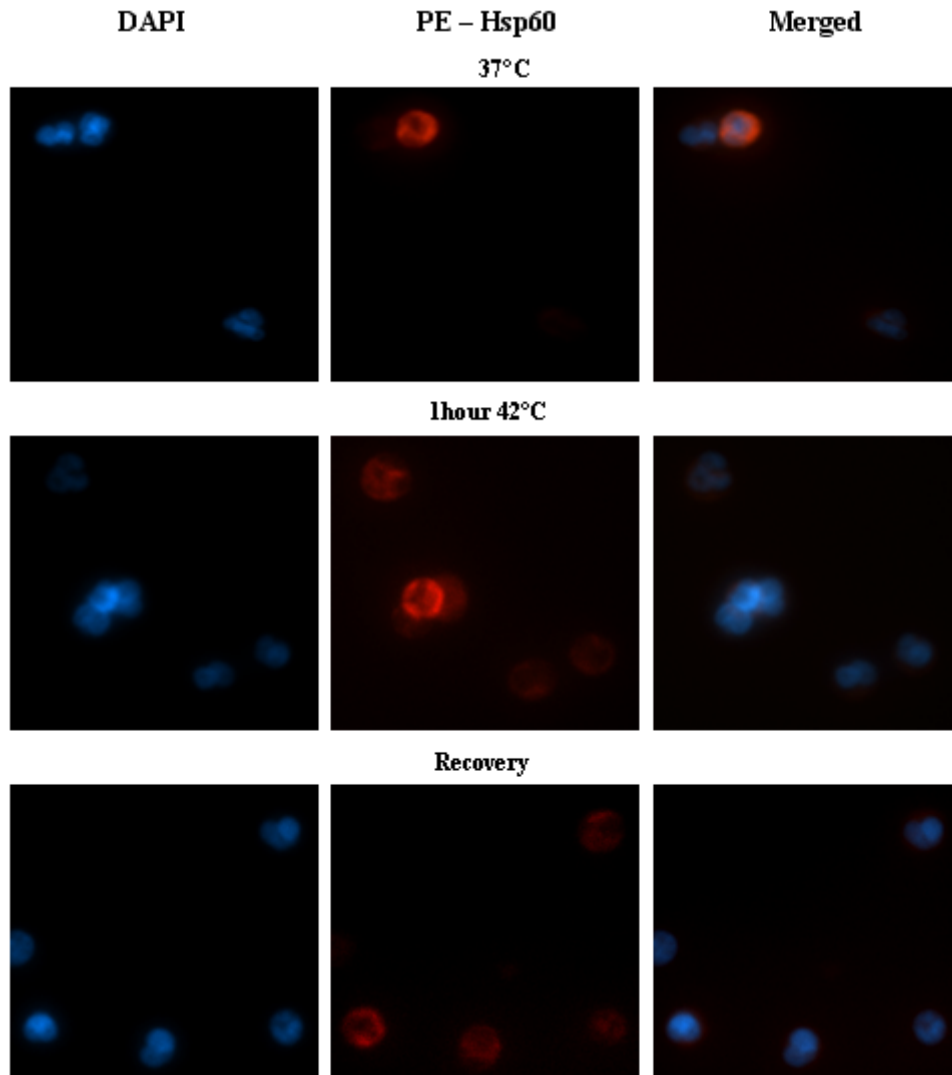
Images were acquired at a magnification of x 60 set up in DAPI (nuclear stain) or TRITC (Hsp60 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



**Figure 3.24: Whole cell Hsp60 in leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 37°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).





**Figure 3.25: Whole cell Hsp60 expression in leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Images were acquired at a magnification of x 60 set up in DAPI (nuclear stain) or TRITC (Hsp60 stain)-fluorescence mode maintained at 25°C on a conventional fluorescence microscope. Data are representative of three independent experiments.



**Figure 3.26: Western blot of membrane associated and whole cell Hsp60 protein from human leukocytes.**

A 10 % gel was cast and loaded with the following under reduced conditions: Lane 1 - leukocyte membrane extract at 37°C; Lane 2 - leukocyte membrane extract heat shocked for 1 hour at 42°C; Lane 3 - leukocyte membrane extract heat shocked and following 3 hours of recovery at 37°C; Lane 4 - pure human Hsp60 (1 µg/mL); Lane 5 - cell lysate at 37°C; Lane 6 - cell lysate heat shocked for 1 hour at 42°C; Lane 7 - cell lysate heat shocked and following 3 hours of recovery at 37°C and Lane 8 - pure human Hsp60 (1 µg/mL). The blot was probed with 1:2000 dilution of anti-Hsp60 followed by anti-mouse IgG peroxidase at 1:5000 dilution, it was stained with SuperSignal® west pico chemiluminescent substrate and exposed for 480 seconds.

### **3.3.6 Expression of surface and whole cell Hsp72 in untreated and heat stressed human leukocytes.**

To further analyse the heat shock response in human leukocytes, the cell surface and whole cell expression of the stress-inducible protein Hsp72 was measured in samples under control conditions and following the heat treatment protocol. The main objective of this part of the study was to investigate the expression of Hsp72 in human leukocytes under control conditions and to determine whether cellular stress leads to altered cell surface and whole cell HSP expression.

The expression of cell surface and whole cell Hsp72 on human leukocytes are presented in Figures 3.27-3.32.

#### **3.3.6.1 Surface and whole cell expression of Hsp72 in human leukocytes.**

The experiments shown in figures 3.28-3.30 reveal human leukocytes to express relatively high base line levels of Hsp72, both on the cell surface (membrane-embedded and membrane-bound) (Figure 3.28) and on/inside (Figure 3.30) human leukocytes under control conditions compared to both Hsp27 and Hsp60. The results suggest the base line level of whole cell Hsp72 to be greater than cell surface (membrane-embedded and membrane-bound) Hsp72 in all leukocyte subtypes ( $P < 0.05$  to  $P < 0.01$  to  $P < 0.001$ , Figures 3.28-3.30). It has previously been demonstrated that the detection of membrane-embedded Hsp72 using the cmHsp72.1 antibody recognises Hsp72 only on abnormal cell cultures, however the results shown in figure 3.28A reveal membrane-embedded Hsp72 on neutrophils and to a lesser extent on lymphocytes and monocytes from normal healthy volunteers', concluding that Hsp72 membrane-embedded expression is not limited to abnormal cell cultures. In comparison, analysis using the alternative anti-human Hsp72 antibody which detects membrane-bound Hsp72 but a different region of the Hsp72 protein, revealed neutrophils and lymphocytes to express lower levels of membrane-bound Hsp72 compared to membrane-embedded expression, whereas monocytes were demonstrated to express significantly higher levels of membrane-bound Hsp72 compared to membrane-embedded expression ( $P < 0.01$ , Figure 3.28A-B). These results indicate Hsp72 is expressed in human leukocytes but its expression varies between the different leukocyte subtypes. Neutrophils ( $P < 0.01$ ) and lymphocytes ( $P < 0.05$ ) were found

to express increased levels of membrane-embedded Hsp72 (Figures 3.28A-B) and monocytes were found to express increased levels of membrane-bound Hsp72 ( $P<0.01$ , Figures 3.28A-B). The difference in the base line levels of Hsp72 appear to be specific for the cell type, overall neutrophils and monocytes expressed considerably higher levels of Hsp72 compared to lymphocytes, which was not related to the number of leukocytes as this was maintained by recording 10,000 events. Consistent with these observations, fluorescence microscopy (Figures 3.29-3.31) showed Hsp72 expressions both on the cell surface and on/inside human leukocytes under control conditions, using the mouse monoclonal anti-human Hsp72: FITC conjugated antibody coupled with DAPI labelling in order to detect the cells nuclei. The results suggested no co-localisation of Hsp72 (green) with the nucleus (blue) in untreated cells. Similarly, the Western blot of human leukocyte membrane extracts and cell lysate probed with a specific antibody for Hsp70 (Figure 3.32) showed bands at approximately 70 kDa in the untreated samples (Lanes 1 and 5).

#### **3.3.6.2 Surface and whole cell expression of Hsp72 in heat stressed human leukocytes.**

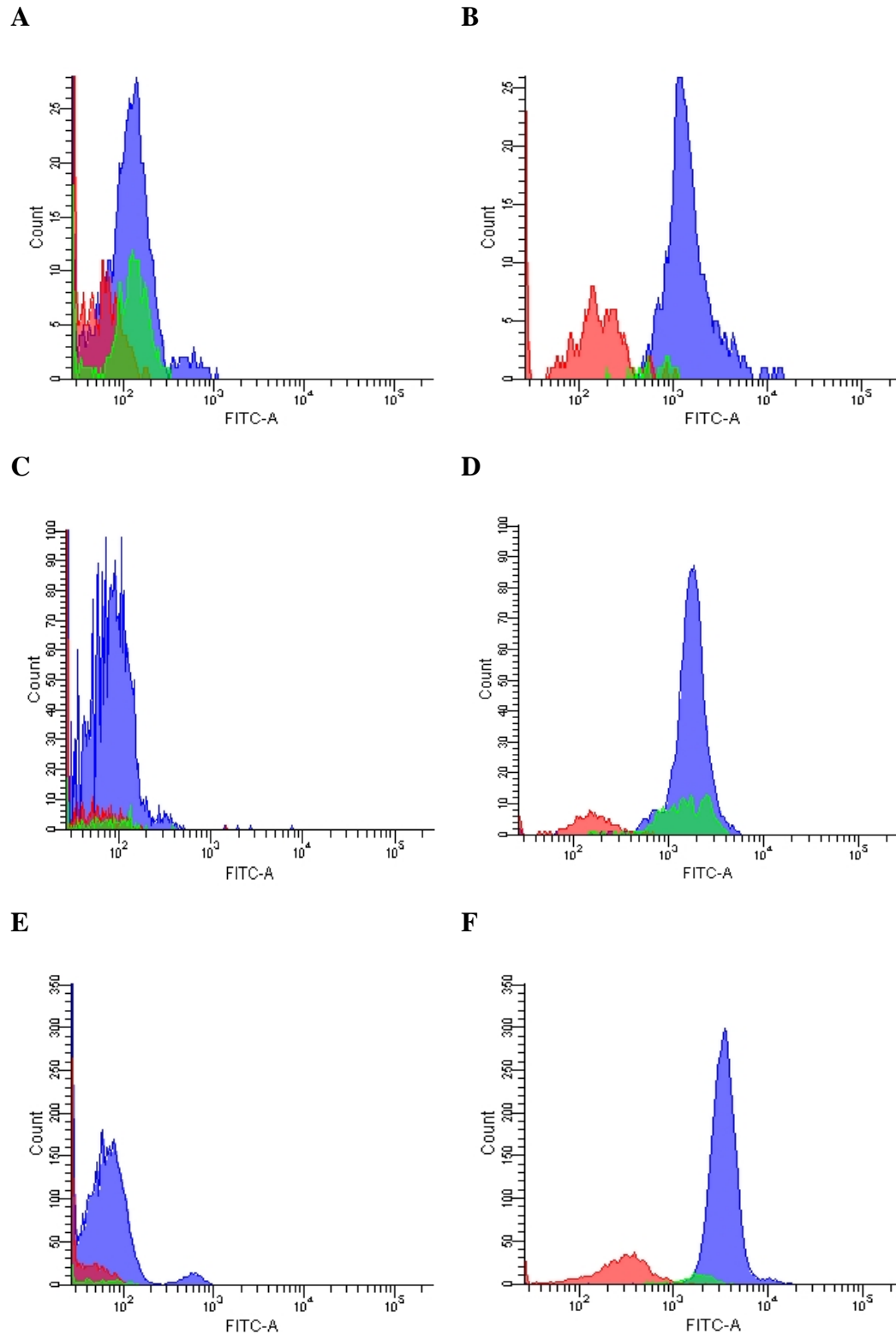
Following incubation of human leukocytes at 42°C for one hour, a significant up-regulation of both cell surface (membrane-embedded and membrane-bound) (Figures 3.28A-B) and whole cell (Figure 3.30) Hsp72 compared to untreated cells occurred. The expression of whole cell Hsp72 was significantly greater than both membrane-embedded and membrane-bound Hsp72 in all leukocyte subtypes ( $P<0.001$ , Figures 3.28-3.30) indicating after heat shock treatment, Hsp72 localisation is mainly concentrated inside human leukocytes. However, the expressions varied amongst the leukocyte subtypes. A 1-fold increase ( $P<0.001$ ) of membrane-bound Hsp72 and an almost 3-fold increase ( $P<0.001$ ) of membrane-embedded Hsp72 was seen compared to untreated controls in neutrophils (Figure 3.28A-B), in addition to an almost 2-fold increase ( $P<0.001$ ) of whole cell Hsp72 (Figure 3.30). Despite the differences in fold-increases the results suggest following heat shock treatment, Hsp72 up-regulation and localisation is mainly concentrated inside neutrophils (Figure 3.28). Lymphocytes also induced a small, yet significant increase in cell surface Hsp72, a 2-fold increase in membrane-bound Hsp72 ( $P<0.001$ ) and a 3-fold increase in

membrane-embedded Hsp72 ( $P<0.001$ ) compared to untreated controls. Similarly, whole cell Hsp72 also increased in lymphocytes, a 2-fold increase ( $P<0.001$ ) compared to untreated cells (Figure 3.30). Monocytes too exhibited a strong induction of Hsp72 following heat stress, an almost 4-fold increase ( $P<0.001$ ) of membrane-bound Hsp72 and a 2-fold increase ( $P<0.001$ ) of membrane-embedded Hsp72 compared to untreated cells (Figure 3.28), in addition to a 1-fold increase ( $P<0.001$ ) of whole cell Hsp72 (Figure 3.30). Again, despite the differences in fold-increases the results suggest following heat shock treatment, Hsp72 up-regulation and localisation is mainly concentrated inside monocytes (Figure 3.28). Neutrophils and monocytes exhibited the highest induction of surface and whole cell Hsp72 following heat shock compared to lymphocytes and untreated samples, however their surface location varied. These results were confirmed by fluorescence microscopy (Figures 3.29-3.31), and revealed increased cell surface and whole cell Hsp72 expression after heat shock treatment compared to untreated cells, but no co-localisation of Hsp72 with the nucleus was observed. In addition, the results of Western blotting (Figure 3.32) also showed increased band intensity at approximately 70 kDa in the samples containing leukocyte membrane extracts and cell lysate that were heat shocked for one hour at 42°C compared to untreated samples (Lanes 2 and 6).

### **3.3.6.3 Surface and whole cell expression of Hsp72 in heat stressed human leukocytes following a three hour recovery at 37°C.**

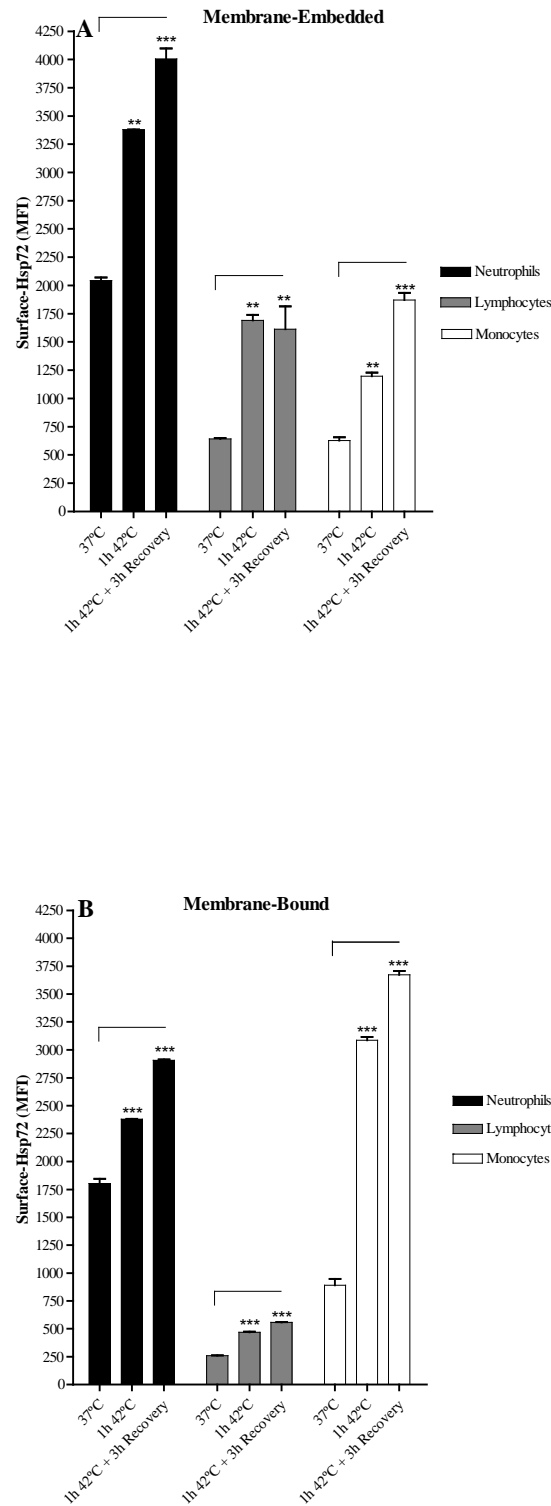
In response to a three hour recovery at 37°C, the expression of whole cell Hsp72 was significantly decreased in all leukocyte subtypes and levels were reduced to values that equalled or were below control levels ( $P<0.001$ , Figure 3.30). Indeed, an almost 2-fold decrease ( $P<0.001$ ) of whole cell Hsp72 was seen in neutrophils compared to the 42°C heat shocked sample (Figure 3.30). Similarly, a 3-fold decrease of whole cell Hsp72 was seen in lymphocytes ( $P<0.001$ ) and a 2-fold decrease in monocytes ( $P<0.001$ ) compared to the 42°C heat shocked samples (Figure 3.30). On the other hand, the decrease of whole cell Hsp72 corresponded with a significant up-regulation of cell surface, both membrane-bound and membrane-embedded Hsp72. Indeed cell surface Hsp72 reached a peak of expression after a three hour post-heat shock in all leukocyte subtypes. A significant increase (1-fold increase,  $P<0.001$ ) in membrane-bound and

membrane-embedded Hsp72 compared to the 42°C heat shocked sample was observed in neutrophils (Figures 3.28A-B), in addition to a 1-fold increase ( $P<0.01$ ) of both membrane-bound and membrane-embedded Hsp72 in lymphocytes (Figures 3.28A-B), and finally an almost 2-fold increase in monocytes ( $P<0.001$ ) of both membrane-bound and membrane-embedded Hsp72 compared to the heat shocked sample (Figures 3.28A-B). These results suggest the movement of Hsp72 within cells and its potential translocation to the cell surface. The results obtained by flow cytometry were again confirmed by fluorescence microscopy (Figures 3.29-3.31), and revealed heat shock significantly enhanced Hsp72 expression and potentiated its translocation, however no evidence for co-localisation of Hsp72 with the nucleus was obtained, suggesting Hsp72 is located within the cytosol or the cell surface. Western blotting (Figure 3.32) showed evidence of increased band intensity at approximately 70 kDa in the samples containing leukocyte membrane extracts and decreased band intensity in the cell lysate that were heat shocked plus recovery for three hours at 37°C compared to the samples that were heat shocked for one hour at 42°C (Lanes 3 and 7).



**Figure 3.27: Surface (A, B using cm.Hsp70.1 and C, D using SPA810) and whole cell (E and F using SPA810) Hsp72 analysis of human leukocytes was performed by flow cytometry.**

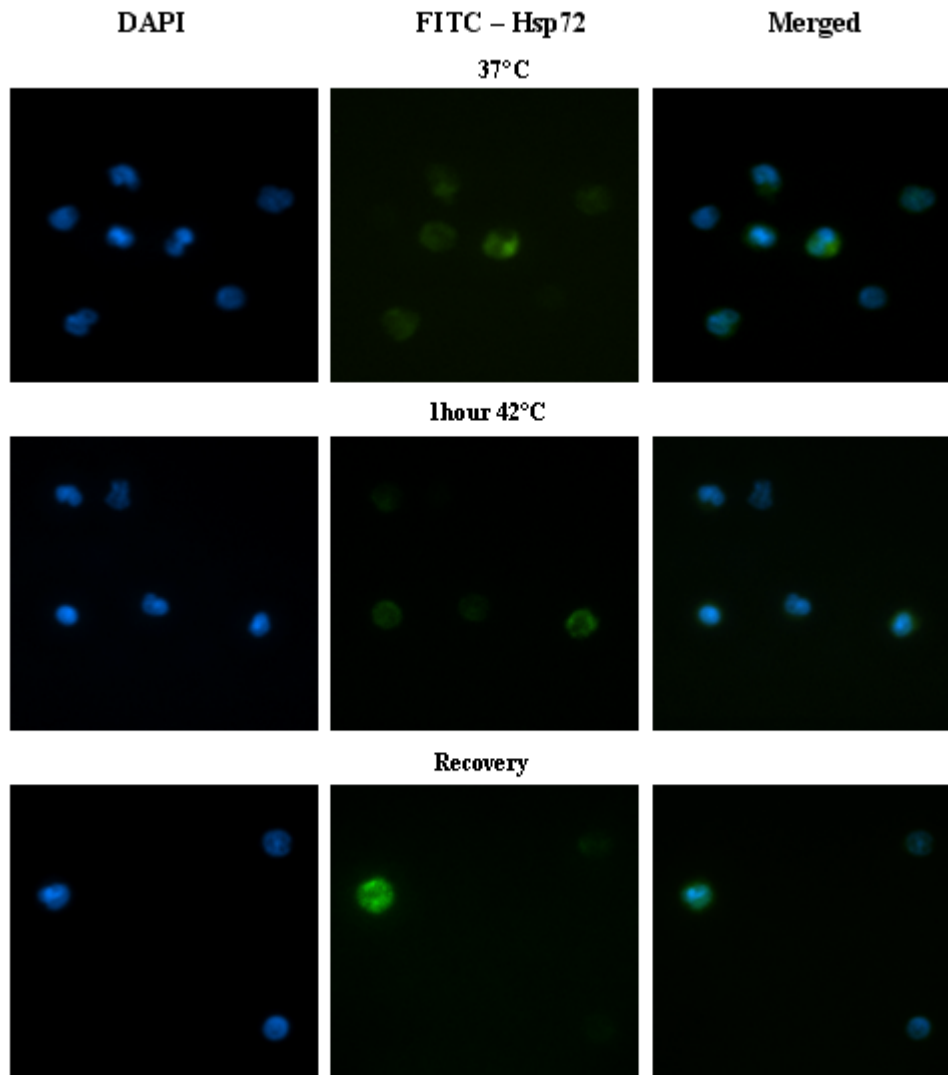
Human leukocytes were treated at 37°C for 1 hour. Neutrophils (■), lymphocytes (■) and monocytes (■) were gated using CD3, CD14, CD15 and FSC/SSC respectively. (A, C, E). No stain control samples. (B, D, F) Hsp72 expression. Data are representative of three independent experiments and 10,000 events were recorded.



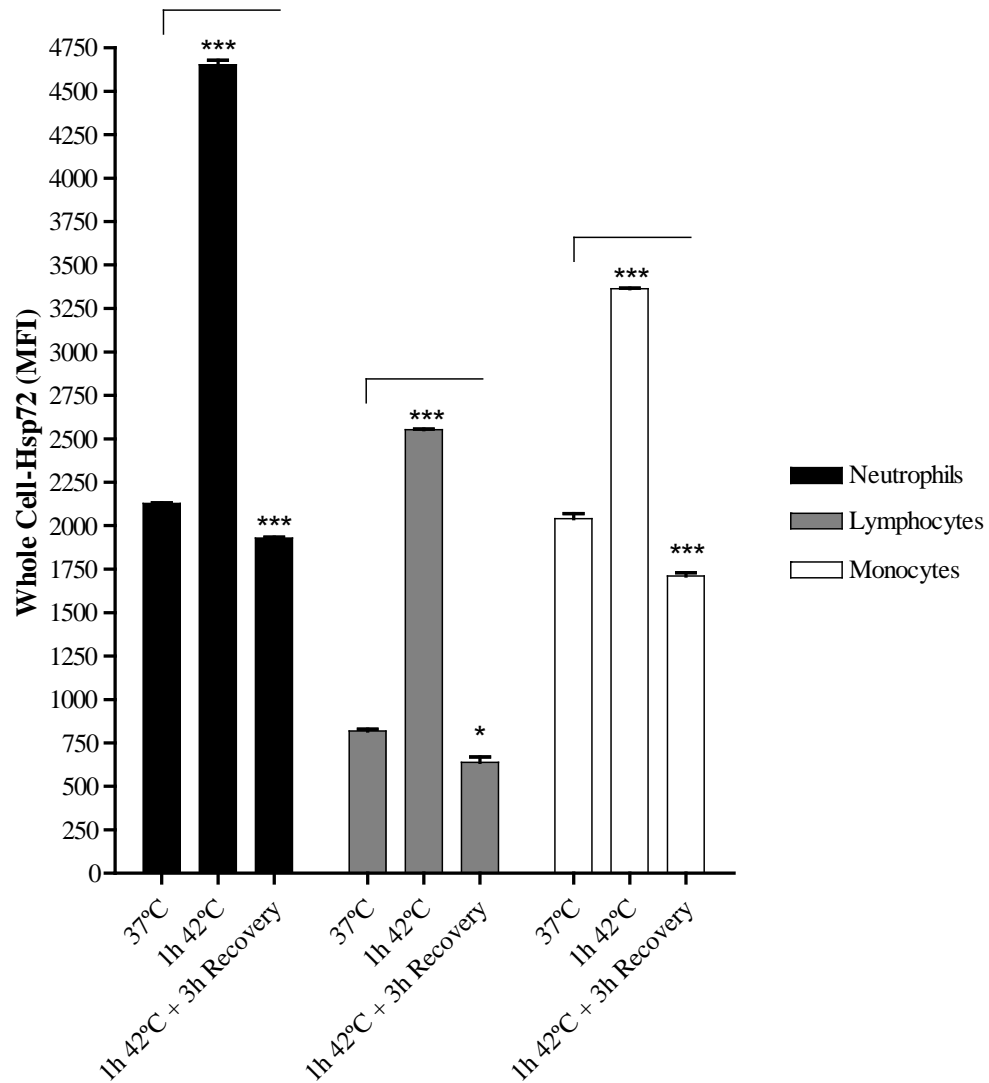
**Figure 3.28: Surface expression of Hsp72 on leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

(A) cm.Hsp70.1 and (B) SPA810. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from 37°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



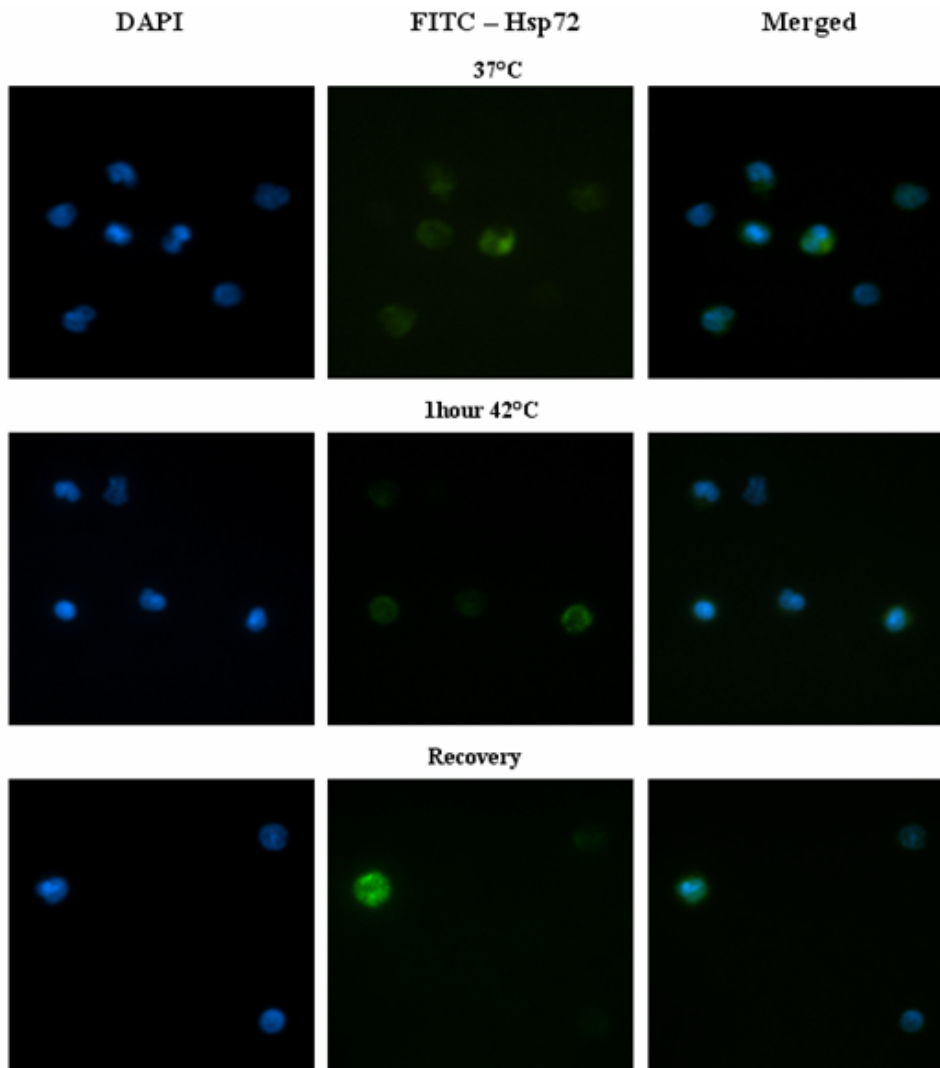


**Figure 3.29: Hsp72 surface expression on leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.** Images were acquired at a magnification of x 60 set up in DAPI (nuclear stain) or FITC (Hsp72 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



**Figure 3.30: Whole cell Hsp72 expression in leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from 37°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\*\* ( $P<0.001$ ).



**Figure 3.31: Whole cell Hsp72 expression in leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Images were acquired at a magnification of x 60 set up in DAPI (nuclear stain) FITC (Hsp72 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



**Figure 3.32: Western blot of membrane associated and whole cell Hsp72 protein from human leukocytes.**

A 10 % gel was cast and loaded with the following under reduced conditions: Lane 1 - leukocyte membrane extract at 37°C; Lane 2 - leukocyte membrane extract heat shocked for 1 hour at 42°C; Lane 3 - leukocyte membrane extract heat shocked and following 3 hours of recovery at 37°C; Lane 4 - pure human Hsp72 (1 µg/mL); Lane 5 - cell lysate at 37°C; Lane 6 - cell lysate heat shocked for 1 hour at 42°C; Lane 7 - cell lysate heat shocked and following 3 hours of recovery at 37°C and Lane 8 - pure human Hsp72 (1 µg/mL). The blot was probed with 1:2000 dilution of biotinylated anti-Hsp72 followed by avidin at 1:5000 dilution, it was stained with SuperSignal® west pico chemiluminescent substrate and exposed for 480 seconds.

### **3.3.7 Expression of surface and whole cell Hsp90 in untreated and heat stressed human leukocytes.**

The findings of the previous sections have suggested that the up-regulation of whole cell HSPs induced by heat shock precedes translocation and cell surface expression in human leukocytes. Thus, the final part of this study analysed in human leukocytes under control conditions and in response to heat shock the expression of cell surface and whole cell Hsp90. The main objective of the final part of the study was to investigate the expression of Hsp90 under normal conditions and to determine whether cellular stress leads to altered HSP expression.

The expression of cell surface and whole cell Hsp90 on human leukocytes are presented in Figures 3.33-3.38.

#### **3.3.7.1 Surface and whole cell expression of Hsp90 in human leukocytes.**

The experiments shown in figures 3.34-3.36 suggest human leukocytes express low base line levels of Hsp90, on the cell surface (Figure 3.34) and on/inside human leukocytes (Figure 3.36) under control conditions. The results revealed human leukocytes to express increased levels of whole cell Hsp90 compared to cell surface levels, neutrophils ( $P<0.001$ ) and lymphocytes ( $P<0.01$ ), however the results for monocytes were found to be non-significant (Figures 3.34-3.36). Between the leukocyte subtypes, neutrophils were found to express increased significant levels of both cell surface and whole cell Hsp90 compared to lymphocytes ( $P<0.001$ , Figures 3.34-3.36) and monocytes ( $P<0.001$ , Figures 3.34-3.36). These results indicate Hsp90 is expressed in human leukocytes but its expression varies between the different leukocyte subtypes. Consistent with these observations, fluorescence microscopy (Figures 3.35-3.37) showed Hsp90 expressions both on the cell surface and on/inside human leukocytes under control conditions using the mouse monoclonal anti-human Hsp90: PE conjugated antibody coupled with DAPI labelling in order to detect the cells nuclei. The results suggested no co-localisation of Hsp90 (red) with the nucleus (blue) in untreated cells. Similarly, the Western blot of human leukocyte membrane extracts and cell lysate probed with a specific antibody for Hsp90

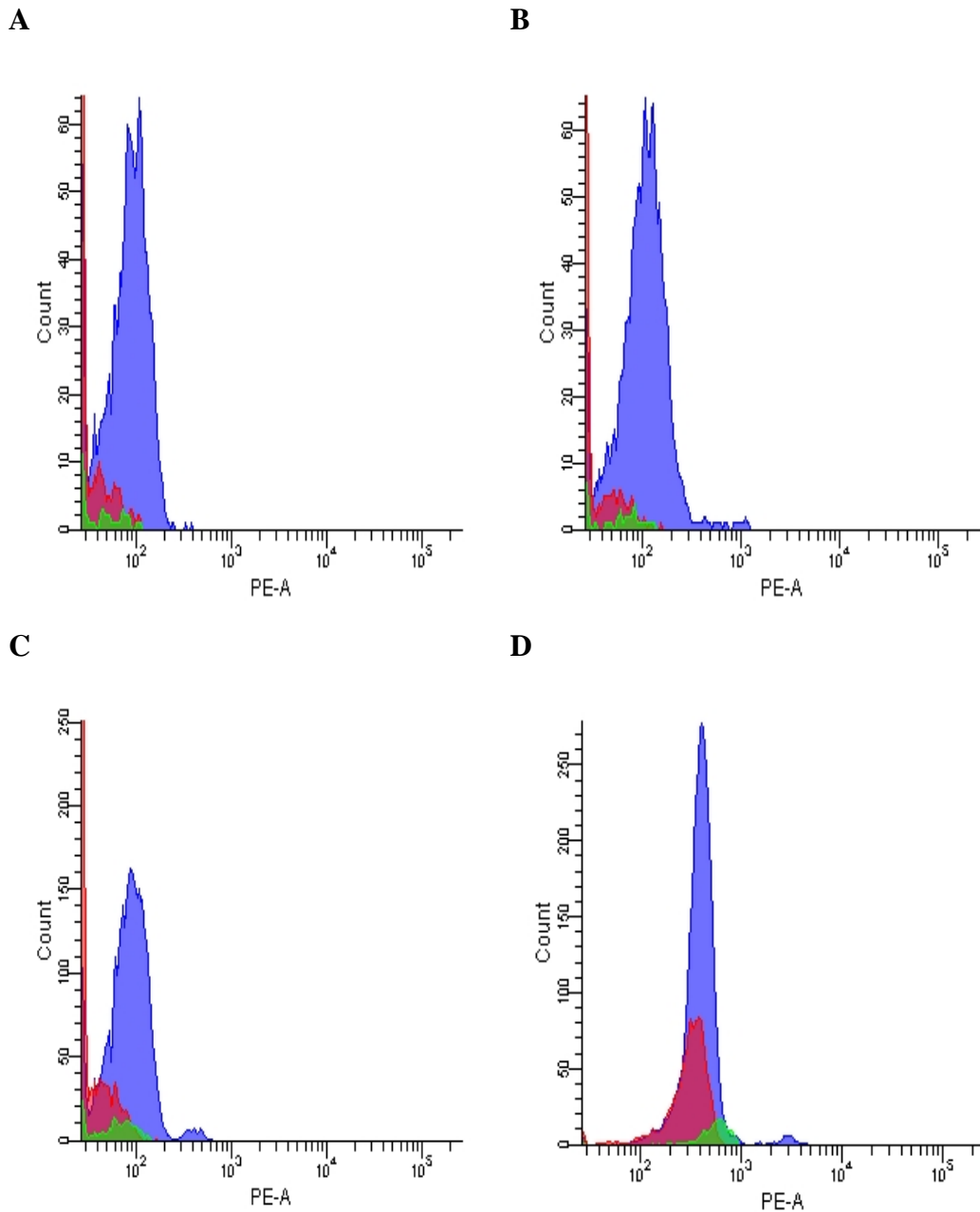
(Figure 3.38) showed bands at approximately 90 kDa in the untreated samples (Lanes 1 and 5).

### **3.3.7.2 Surface and whole cell expression of Hsp90 in heat stressed human leukocytes.**

The exposure of human leukocytes to an elevated temperature of 42°C for one hour, led to significant changes in the level of expression of cell surface (Figure 3.34) and whole cell (Figure 3.36) Hsp90 compared to untreated cells. Indeed, a significant up-regulation of both cell surface ( $P<0.001$ , Figure 3.34) and whole cell ( $P<0.001$ , Figure 3.36) Hsp90 resulted. However, the expression was found to vary between the leukocytes subtypes. A 4-fold increase ( $P<0.001$ ) of cell surface Hsp90 was seen compared to untreated controls in neutrophils (Figure 3.34), in addition to an almost 5-fold increase ( $P<0.001$ ) of whole cell Hsp90 (Figure 3.36). In contrast, in monocytes an 8-fold increase ( $P<0.001$ ) of cell surface Hsp90 was seen compared to untreated controls (Figure 3.34), in addition to an almost 14-fold increase ( $P<0.001$ ) of whole cell Hsp90 (Figure 3.36). Despite the difference in fold increase, the up-regulation of Hsp90 in neutrophils was found to be significantly greater than monocytes ( $P<0.001$ , Figures 3.34-3.36). These results suggest following heat shock treatment, Hsp90 up-regulation and localisation is mainly concentrated inside neutrophils and monocytes (Figure 3.34). In comparison, lymphocytes exhibited an extremely significant up-regulation of cell surface Hsp90, a 15-fold increase ( $P<0.001$ , Figure 3.34) and an almost 13-fold increase of whole cell Hsp90 ( $P<0.001$ , Figure 3.36) compared to untreated cells. However, the expressions did not vary between cell surface and whole cell location, although a significant increase of Hsp90 expression was observed ( $P<0.001$ , Figures 3.34-3.36), but the MFI levels were lower compared to both neutrophils and monocytes. These results were confirmed by fluorescence microscopy (Figures 3.35-3.37), and revealed increased cell surface and whole cell Hsp90 expression after heat shock treatment compared to control cells, but no co-localisation of Hsp90 with the nucleus was observed. In addition, the results of Western blotting (Figure 3.38) also showed increased band intensity at approximately 90 kDa in the samples containing leukocyte membrane extracts and cell lysate heat shocked for one hour at 42°C compared to untreated samples (Lanes 2 and 6).

### **3.3.7.3 Surface and whole cell expression of Hsp90 in heat stressed human leukocytes following a three hour recovery at 37°C.**

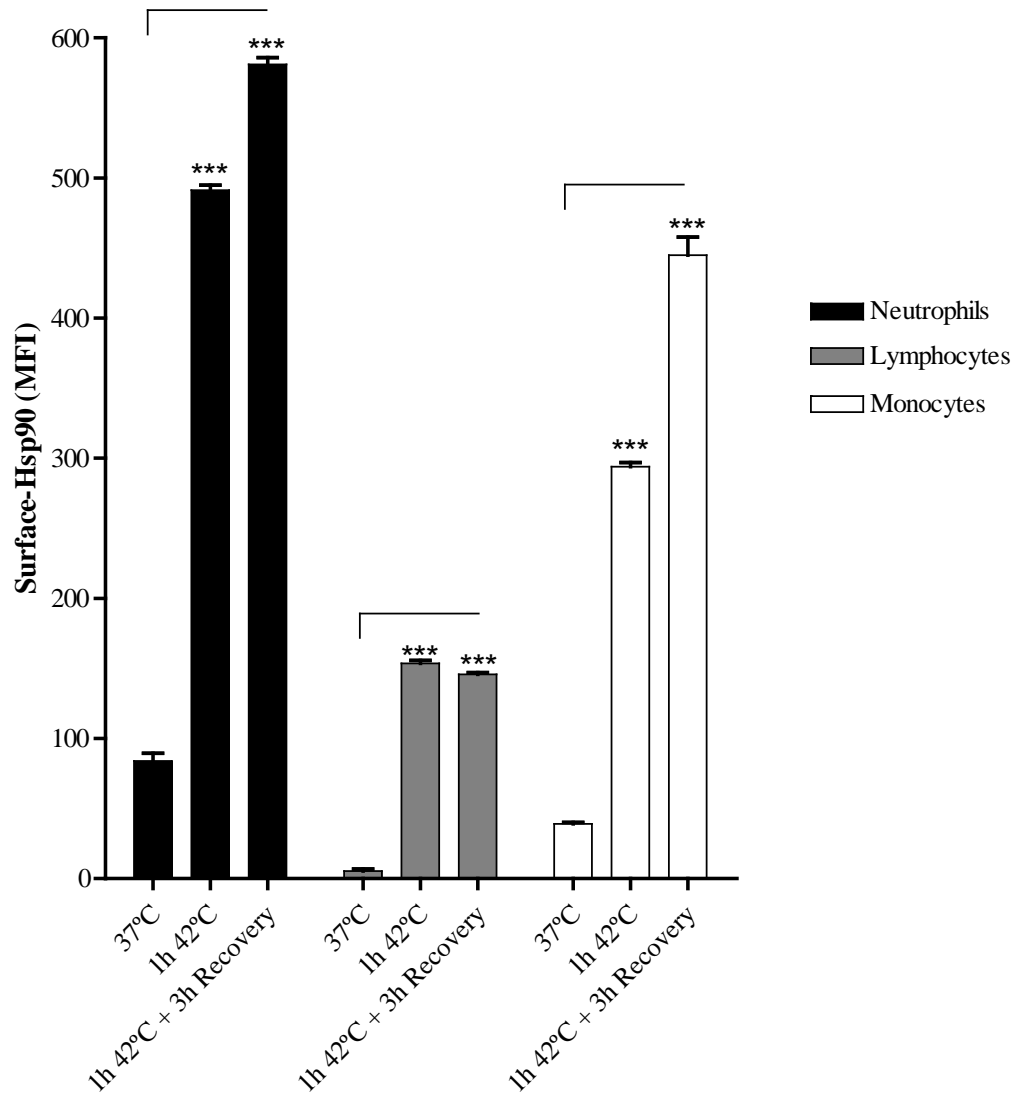
Following recovery after a three hour culture, the expression of whole cell Hsp90 was slightly down-regulated. An almost 1-fold decrease of whole cell Hsp90 was seen in neutrophils compared to the 42°C heat shocked sample (Figure 3.36). Similarly, a 1-fold decrease of whole cell Hsp90 was also seen in both lymphocytes and monocytes compared to the 42°C heat shocked sample (Figure 3.36), yet despite these fold decreases the results were found to be non-significant (Figure 3.36) and levels did not decrease to equal or below the values of the untreated controls (Figure 3.36). These results corresponded with a significant increase in the surface expression of Hsp90 compared to control cells. A 1-fold increase ( $P < 0.001$ ) of cell surface Hsp90 was seen compared to the 42°C heat shocked sample in neutrophils and monocytes, however lymphocytes resulted with a non-significant difference (Figure 3.34). These results suggest the movement of Hsp90 within cells and its potential translocation to the cell surface. The results obtained by flow cytometry were confirmed by fluorescence microscopy (Figures 3.35-3.37), and revealed heat shock significantly enhanced Hsp90 expression and potentiated its translocation, however no evidence for co-localisation of Hsp90 with the nucleus was obtained, suggesting Hsp90 must be located within the cytosol or the cell surface. Western blotting (Figure 3.38) showed evidence of increased band intensity at approximately 90 kDa in the samples containing leukocyte membrane extracts and decreased band intensity in cell lysate heat shocked plus recovery for three hours at 37°C compared to the samples heat shocked for one hour at 42°C (Lanes 3 and 7).



**Figure 3.33: Surface (A and B) and whole cell (C and D) Hsp90 analysis of human leukocytes was performed using flow cytometry.**

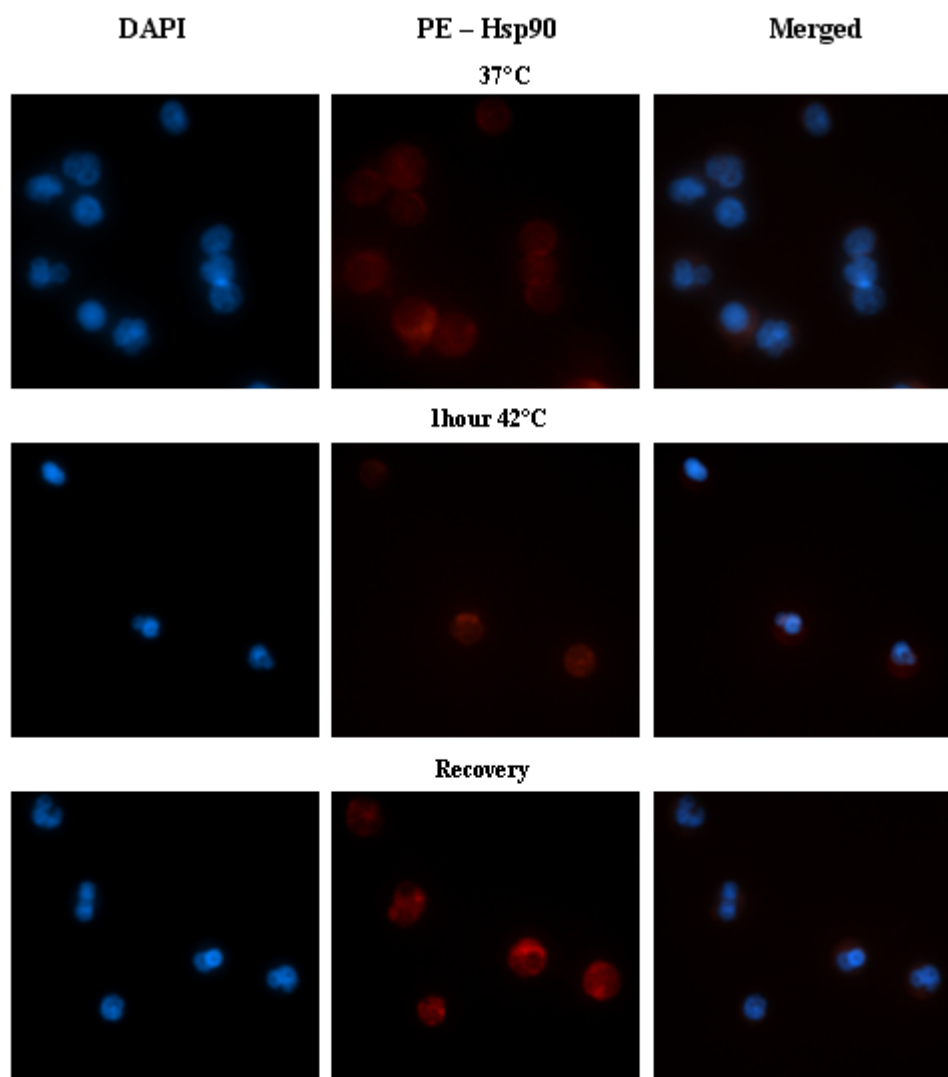
Human leukocytes were treated at 37°C for 1 hour. Neutrophils (■), lymphocytes (■) and monocytes (■) were gated using CD3, CD14 and CD15 and FSC/SSC respectively. (A, C) No stain control samples. (B, D) Hsp90 expression. Data are representative of three independent experiments and 10,000 events were recorded.





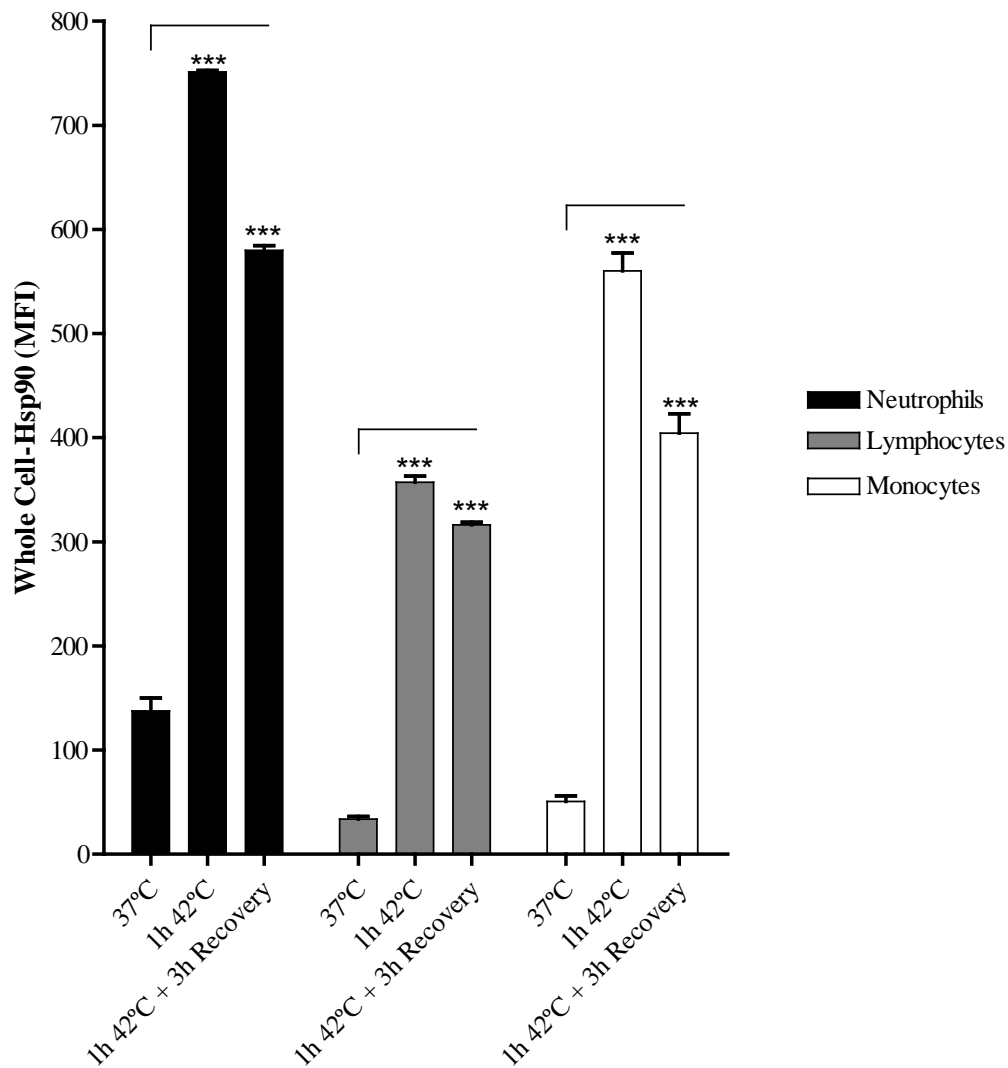
**Figure 3.34: Surface expression of Hsp90 on leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 37°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).



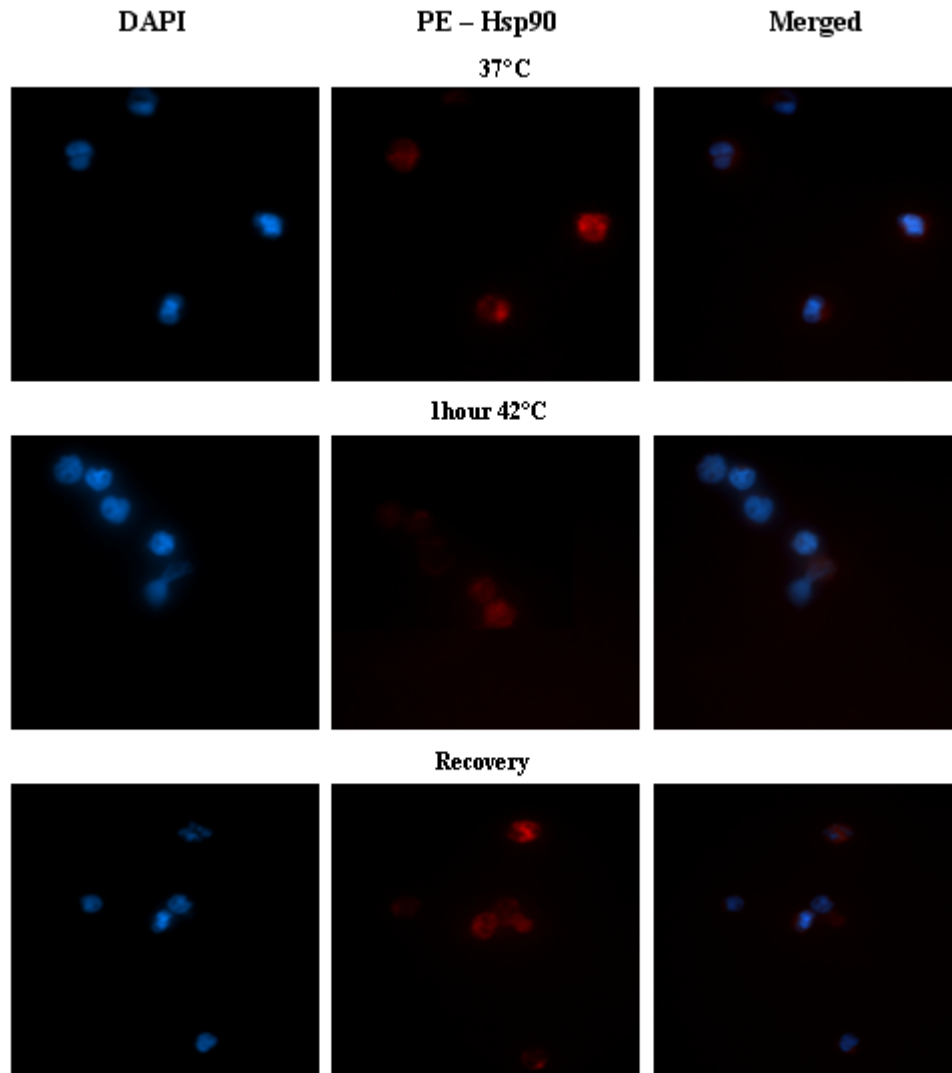
**Figure 3.35: Surface expression of Hsp90 on leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Images were acquired at a magnification of x 60 set up in DAPI (nuclear stain) or TRITC (Hsp90 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



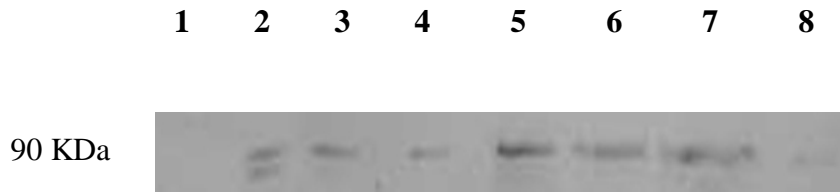
**Figure 3.36: Whole cell Hsp90 expression in leukocytes at 37°C, in response to 1 hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from 37°C between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



**Figure 3.37: Whole cell Hsp90 expression in leukocytes at 37°C, in response to an hour heat shock treatment (42°C) and following 3 hour recovery at 37°C.**

Images were acquired at a magnification of x 60 set up in DAPI (nuclear stain) or TRITC (Hsp90 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments



**Figure 3.38: Western blot of membrane associated and whole cell Hsp90 protein from human leukocytes.**

A 10 % gel was cast and loaded with the following under reduced conditions: Lane 1 - leukocyte membrane extract at 37°C; Lane 2 - leukocyte membrane extract heat shocked for 1 hour at 42°C; Lane 3 - leukocyte membrane extract heat shocked and following 3 hours of recovery at 37°C; Lane 4 - pure human Hsp90 (1 µg/mL); Lane 5 - cell lysate at 37°C; Lane 6 - cell lysate heat shocked for 1 hour at 42°C; Lane 7 - cell lysate heat shocked and following 3 hours of recovery at 37°C and Lane 8 - pure human Hsp90 (1 µg/mL). The blot was probed with 1:2000 dilution of anti-Hsp90 followed by anti-mouse IgG peroxidase at 1:5000 dilution, it was stained with SuperSignal® west pico chemiluminescent substrate and exposed for 480 seconds.

### **3.3.8 Expression of surface HSPs in untreated and heat stressed human leukocytes following incubation with NaCl.**

The preceding observations suggest HSPs are not only expressed inside human leukocytes but are capable of interacting with the cell surface, membrane-bound and/or membrane-embedded. Thus, a series of experiments were performed involving the treatment of human leukocytes with high-salt concentrations (2 minute incubation with 0-40 mM). The expression of Hsp27, Hsp60, Hsp72 and Hsp90 on the cell surface of control and heat shocked human leukocytes in response to NaCl treatment was determined quantitatively by flow cytometry. The main objective of this part of the study was to investigate cell surface (membrane-bound) HSP expression on human leukocytes.

The expression of cell surface HSPs on human leukocytes are presented in Figures 3.39-3.42.

#### **3.3.8.1 Surface (membrane-bound) expression of HSPs in untreated human leukocytes following incubation with NaCl.**

As previously determined (Figures 3.16, 3.24, 3.28, 3.34) human leukocytes express base line levels of cell surface HSPs under control conditions and are significantly up-regulated when stressed by elevated temperatures ( $P < 0.001$ ). Treatment with increasing concentrations of NaCl demonstrated decreased antibody recognition when analysing surface Hsp27, Hsp60, Hsp72 and Hsp90, indicating that the HSPs being detected by these antibodies can be 'washed off' and therefore it can be hypothesised that the HSPs can be easily removed from the cell surface. It was again apparent that all surface HSP expressions varied between the leukocyte subtypes.

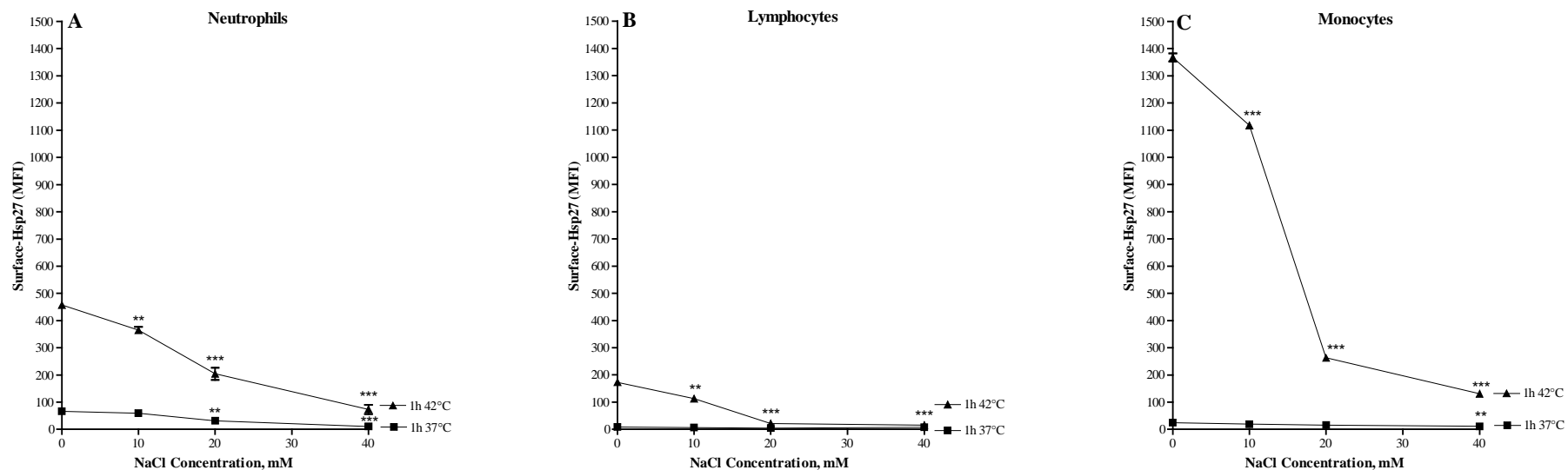
#### **3.3.8.2 Surface (membrane-bound) expression of HSPs in heat stressed human leukocytes following incubation with NaCl.**

The experiments shown in figure 3.39A-C reveal all three leukocyte subtypes to have similar surface (membrane-bound) HSP expressions when treated with increasing concentrations of NaCl. 10 mM NaCl significantly reduced Hsp27 ( $P < 0.01$ , Figure 3.39A,  $P < 0.01$ , Figure 3.39B and  $P < 0.001$ , Figure 3.39C) membrane-bound expression compared to the heat shocked control. As the

concentration of NaCl increased, the expression of Hsp27 significantly decreased ( $P<0.001$ ) to levels which were equal to the untreated control (Figure 3.39A-B), with the exception of monocytes (Figure 3.39C). In addition it was also found, similar to Hsp27 expression, all three leukocyte subtypes to have similar surface Hsp60 expressions when treated with NaCl as shown in figure 3.40A-C. However, treatment with 10 mM NaCl did not cause a significant decrease in membrane-bound Hsp60 (Figure 3.40A-C) compared to the heat shocked control in any of the leukocyte subtypes. Yet, treatment with 20 mM NaCl did significantly decrease membrane-bound Hsp60 ( $P<0.05$ , Figure 3.40A,  $P<0.05$ , Figure 3.40B and  $P<0.01$ , Figure 3.40C) compared to the heat shocked control. Furthermore, treatment with 40 mM NaCl decreased membrane-bound Hsp60 ( $P<0.01$ , Figure 3.40A,  $P<0.001$ , Figure 3.40B and  $P<0.001$ , Figure 3.40C) compared to the heat shocked control and lymphocytes reduced their Hsp60 membrane-bound expression to levels which were equal to the values of the untreated control (Figure 3.40B). A significant decrease ( $P<0.05$  to  $P<0.01$  to  $P<0.001$ ) of membrane-bound Hsp72 was also observed following treatment with NaCl (Figure 3.41A-C) in all three leukocyte subtypes. However, neutrophils required treatment with the increased concentration of 20 mM NaCl for a significant decrease in membrane-bound Hsp72 ( $P<0.001$ , Figure 3.41A) to be observed compared to the heat shocked control. At concentrations lower than this (10 mM) a non-significant difference from the heat shocked control was observed (Figure 3.41A). In contrast, treatment with 10 mM NaCl significantly decreased membrane-bound Hsp72 on both lymphocytes ( $P<0.001$ , Figure 3.41B) and monocytes ( $P<0.05$ , Figure 3.41C) compared to the heat shocked control. In addition, treatment of human leukocytes with 40 mM NaCl similar to previous findings (Figures 3.39-3.40) decreased significantly Hsp72 membrane-bound expression on all three leukocyte subtypes compared to the heat shocked control ( $P<0.001$ , Figure 3.41A-C), and again in lymphocytes to levels which were equal to that of the untreated control (Figure 3.41B). Finally, when analysing the membrane-bound expression of Hsp90 as shown in figure 3.42, human leukocytes incubated with NaCl observed a significant decrease ( $P<0.05$  to  $P<0.01$  to  $P<0.001$ ) of the surface expression of Hsp90 compared to the heat shocked control (Figure 4.42A-C). 10 mM NaCl significantly decreased membrane-bound Hsp90 expression on neutrophils ( $P<0.001$ , Figure 3.42A),

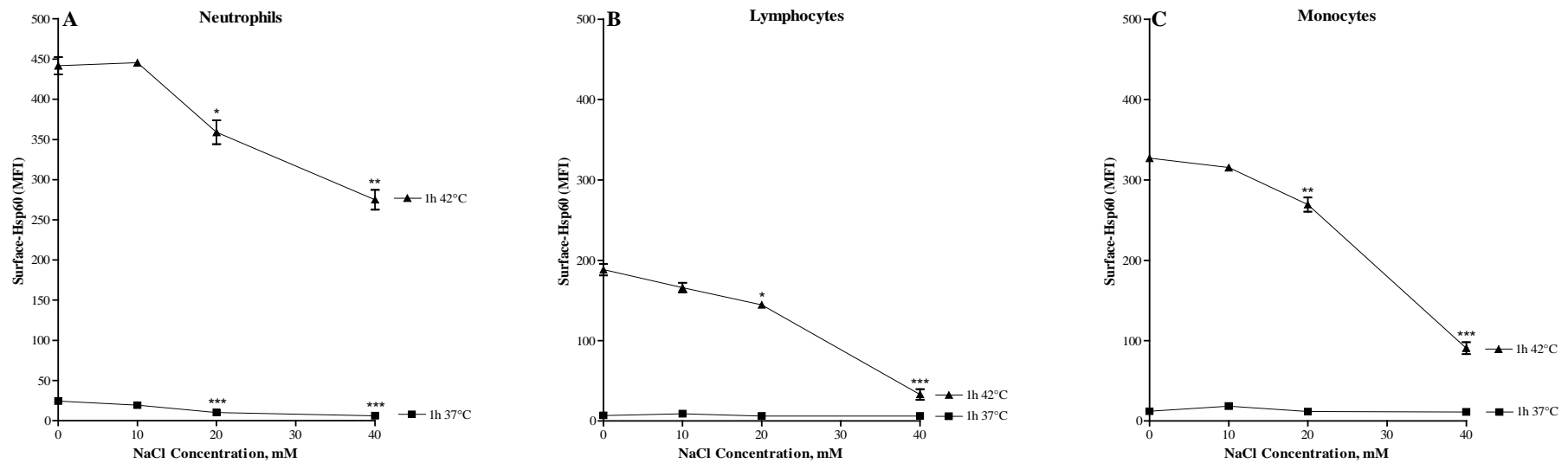
however membrane-bound levels of Hsp90 on both lymphocytes and monocytes were not significantly different from the heat shocked control (Figure 3.42B-C). An increased concentration of 20 mM NaCl was required for a significant decrease to be observed in the membrane-bound expression of Hsp90 ( $P < 0.05$ , Figure 3.42B and  $P < 0.001$ , figure 3.42C) on these cell types. Again, incubation of human leukocytes with 40 mM NaCl caused a significant decrease ( $P < 0.01$ , Figure 3.42A,  $P < 0.01$ , Figure 3.42B and  $P < 0.001$ , Figure 3.42C) of membrane-bound Hsp90 on all three leukocyte subtypes to levels which were significantly different from the heat shocked control (Figure 3.42A-C)





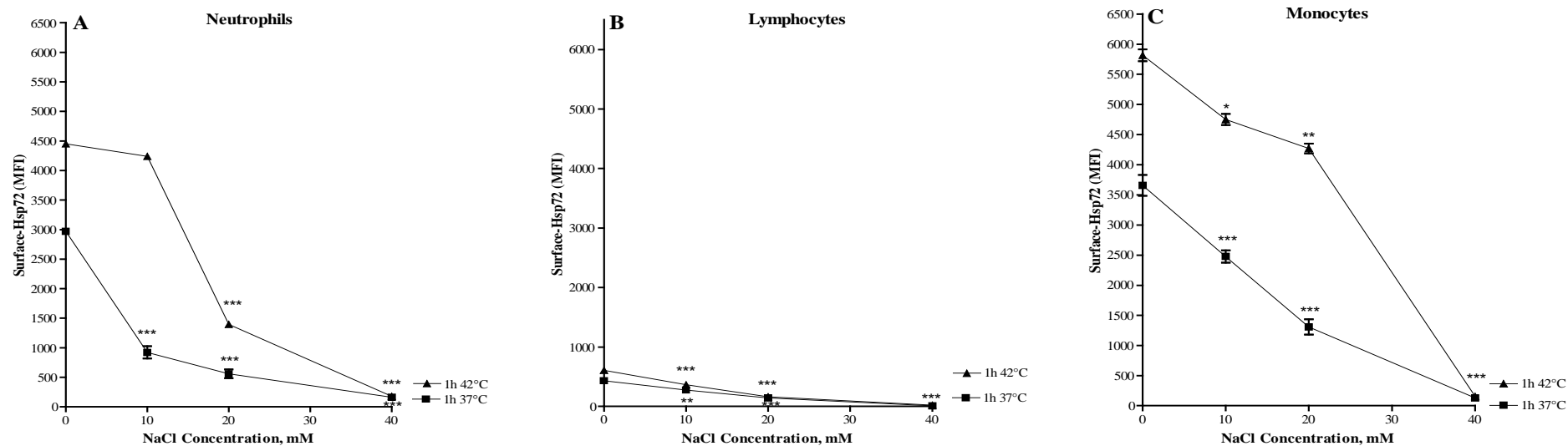
**Figure 3.39: Surface expression of Hsp27 on untreated (37°C) and heat shocked (42°C) human leukocytes following treatment with 0-40 mM NaCl.**

Human leukocytes were incubated at 37°C or 42°C for 1 hour followed by treatment with NaCl for 2 minutes at RT. Normal physiological concentration of NaCl in the body is in the range of 136-146 mM. Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 0 mM control between mean concentration points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* (P<0.01), \*\*\* (P<0.001).



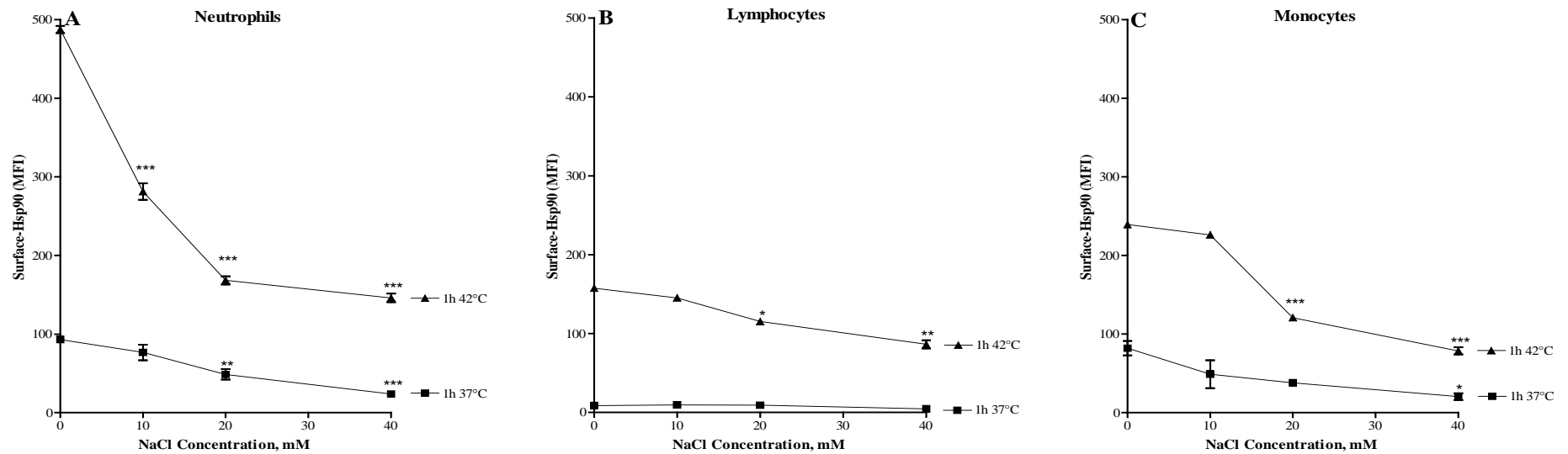
**Figure 3.40: Surface expression of Hsp60 on untreated (37°C) and heat shocked (42°C) human leukocytes following treatment with 0-40 mM NaCl.**

Human leukocytes were incubated at 37°C or 42°C for 1 hour followed by treatment with NaCl for 2 minutes at RT. Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 0 mM control between mean concentration points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001).



**Figure 3.41: Surface expression of Hsp72 on untreated (37°C) and heat shocked (42°C) human leukocytes following treatment with 0-40 mM NaCl.**

Human leukocytes were incubated at 37°C or 42°C for 1 hour followed by treatment with NaCl for 2 minutes at RT. Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 0 mM control between mean concentration points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001).



**Figure 4.42: Surface expression of Hsp90 on untreated (37°C) and heat shocked (42°C) human leukocytes following treatment with 0-40 mM NaCl.**

Human leukocytes were incubated at 37°C or 42°C for 1 hour followed by treatment with NaCl for 2 minutes at RT. Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 0 mM control between mean concentration points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001).

### **3.4 Discussion**

The aim of this chapter was to determine the effects of *in vitro* elevated temperature stress in U937 monocytes and human leukocytes. It also aimed to explore the up-regulation, localisation and movement of HSPs, and investigated the role of HSPs in apoptosis and necrosis through examining a potential relationship between heat-induced cell death and HSP translocation to the cell surface.

#### **3.4.1 Temperature induces apoptosis and necrosis in different cell types.**

It was shown that cells exposed to elevated temperatures of 37.7°C-46.2°C for up to six hours induced both apoptosis and necrosis in a temperature and time-dependent manner and decreased cell viability in all cell types studied. However, the induction was found to be cell specific. U937 monocytes underwent apoptosis at the sub-lethal temperature of 40.8°C, where a peak of caspase-3 activity was observed after three hours. At exposure times prior to this, intracellular HSP expression may have protected U937 cells from heat-induced apoptosis through producing a state of acquired thermotolerance. This has been suggested by Wang & Borkan, (1996) and Mosser et al. (2000), who describe the elevated expression of intracellular Hsp72 induced by a mild heat stress to provide cells with an increased level of resistance to a more lethal heat treatment that would normally result in apoptosis. Furthermore, necrosis was induced in U937 cells using a more severe temperature of 46.2°C after a prolonged time period of six hours, which corresponded with a significant decrease of U937 cell viability. These results are in agreement with previous findings which indicate low temperatures induce apoptosis through the intrinsic pathway whilst increasing temperatures induce cell death by necrosis (Mosser et al. 1997; Sapozhnikov et al. 1999; Vacca et al. 2004; Zhou et al. 2005), this suggests that some of the early events involved in heat-induced cell death may be common to both forms, apoptotic and necrotic cell death. Human leukocytes, specifically monocytes, expressed a response which was similar to that of U937 cells. Treatment at the sub-lethal temperature of 40.8°C induced a peak of caspase-3 activity after a three hour exposure. Here, HSPs may not have been sufficient to rescue these cells from heat-induced cell death because pro-apoptotic pathways predominated or because HSPs effects were antagonized or possibly because

monocytes were not activated, the heat shock treatment may not have produced a sufficient signal(s) to stimulate the activation and function of these cells and thus were not required within the innate immune response. In contrast, neutrophils and lymphocytes induced a peak of caspase-3 activity at the increased temperature of 42.7°C after a two to three hour exposure. The increased level of resistance of these cells to sub-lethal temperatures may be the result of the induction of intracellular HSPs at lower temperatures and exposure times which can afford some protection at the early stages of heat-induced cell damage. HSPs may function to protect these cells because of their importance within the immune response. Neutrophils are one of the first-responders to cellular stress which function as professional phagocytes within the innate immune response and lymphocytes within the adaptive immune response, but HSPs are unable to provide complete protection once an apoptotic cascade has been triggered (Samali & Cotter, 1996). However, in response to the more severe temperature of 44.4°C after a four hour exposure, in each leukocyte subtype an increase in necrotic cell death was seen. Thus, it appears similar to the results obtained with U937 cells, at low temperatures apoptosis is the mode of cell death, through the activation of pro-apoptotic members of the Bcl-2 family which function in the intrinsic pathway, stimulating caspase activation and release of cytochrome *c* (Mosser et al. 2000). Through increasing the temperature and exposure time switches the mode of cell death to necrosis along with a decrease of cell viability. A rise in temperature decreases the viability of cells for several reasons including, cytotoxic effects, denaturation of cytoplasmic and membrane proteins and the inhibition of DNA, RNA and protein synthesis (Hildebrandt et al. 2002).

### **3.4.2 Temperature induces HSP expression in different cell types.**

Cells exposed to the elevated temperature of 42°C for one hour were found to have increased expressions of whole cell (surface and intracellular in permeabilized cells) HSPs (Hsp27, Hsp60, Hsp72 and Hsp90) in a time-dependent and cell specific manner compared to untreated controls. Previous studies have shown that the time course of heat shock treatment and recovery used in this chapter was appropriate for intracellular HSP up-regulation (Wang & Edens, 1998; Oehler et al. 2001). Hsp72 expression was significantly up-regulated following a one hour heat shock treatment at 42°C compared to

untreated controls in all cells studied and the other HSPs were also detected but to a lesser degree. Despite the increased expressions of whole cell HSPs in all cells studied, the expressions were found to vary between individual cell types. Firstly, it was found that an elevated temperature of 42°C increased the whole cell expression of Hsp72 in U937 cells compared to untreated controls which is in agreement with another study (Galea-Lauri et al. 1996). Furthermore, Hsp72 expression was also induced in human leukocytes, consistently both neutrophils and monocytes up-regulated a significant increased level of whole cell Hsp72 compared to lymphocytes. In addition, neutrophils up-regulated a significant increased level of whole cell Hsp72 compared to both lymphocytes and monocytes. This is in agreement with earlier observations (Fincato et al. 1991; Polla et al. 1996; Fehrenbach et al. 2000; Oehler et al. 2001; Lim et al. 2005), who established isolated neutrophils and monocytes to induce appreciable levels of Hsp72 mRNA to a different extent in response to exogenous stimuli, whilst transcription was nearly absent in lymphocytes. Furthermore, Dressel & Gunther (1999) found induction of Hsp72 in response to *in vitro* heat shock of isolated human lymphocytes does not occur in all cells and occurs to a varying degree. The up-regulation of the whole cell expression of Hsp72 in response to elevated temperature stress is suggested to be a protective mechanism, since Hsp72 has been previously shown to inhibit the process of heat-induced apoptosis (Garrido et al. 2001; Stankiewicz et al. 2005). Hsp72 protection is thought to occur downstream of cytochrome *c* release (Jäättelä et al. 1998; Li et al. 2000), upstream or downstream of caspase activation (Li et al. 2000) and be dependent on or independent of its ability to inhibit JNK activity within the intrinsic pathway (Mosser et al. 1997). In addition, it has also been suggested that an interaction of Hsp72 with the anti-apoptotic proteins Bcl<sub>2</sub> or Bcl-XL could increase the resistance of cells to heat-induced apoptosis through inhibiting mitochondrial events early in the process of the intrinsic pathway (Wang & Borkan, 1996). Hsp72 may function to protect human leukocytes because of the importance of these cells within the innate and adaptive immune responses. HSPs may enable these cells to survive long enough to fulfil their major functions, as professional killers (neutrophils), antigen processing and presentation (monocytes) and cell-mediated immunity (lymphocytes), before heat-induced cell death.

A one hour heat shock treatment was also able to stimulate Hsp27, Hsp60, as well as Hsp90 expression in human leukocytes, however there was a reduced up-regulation when compared to Hsp72. Neutrophils up-regulated a significant level of whole cell Hsp27, Hsp60 and Hsp90 compared untreated controls. Furthermore, the expressions were greater than both whole cell levels detected in lymphocytes and monocytes. These results are in agreement with previous studies (Arrigo et al. 2004; Bethke et al. 2002; Galea-Lauri et al. 1996). Similar to Hsp72 whole cell expression it has also been suggested that an over-expression of Hsp27 and Hsp90 protects cells against apoptotic cell death induced by hyperthermia (Schmitt et al. 2007), through inhibiting the activation of caspases, the relay of signalling from cell surface receptors (DISC formation), and the release of cytochrome *c* upstream of the mitochondria (Garrido et al. 2001) or because of a negative effect on Apaf-1 function (apoptosome formation) (Pandey et al. 2000b). However, an earlier study has also revealed that the intracellular expression of Hsp90 induced by heat shock is associated with increased apoptosis (Galea-Lauri et al. 1996). This may be due to the role of Hsp90 as a molecular chaperone, in which Hsp90 may chaperone a protein or lipid that is implicated in a signal transduction pathway linked to apoptosis. Thus, it appears conflicting evidence exists as to the function of intracellular HSPs induced by heat shock treatment. Furthermore, the over-expression of intracellular Hsp60 is proposed to be associated with activating the innate immune response (Bethke et al. 2002), this would appear appropriate in this study where cells implicated in the immune response were analysed. Thus, in response to an elevated temperature stress, intracellular Hsp60 may be up-regulated in human leukocytes in order to induce an immune response against the effects of cellular stress. Since the body temperature rarely exceeds 41°C during illness, it cannot be concluded that the HSP induction observed at 42°C also occurs under physiological conditions. Nevertheless, it can be said HSP induction in the leukocyte subsets reflects a cell type specific variation of the stress response. It was also observed that cells incubated for three hours at 37°C, following exposure to elevated temperature stress at 42°C, had an induced, significant expression of whole cell HSPs (Hsp27, Hsp60, Hsp72 and Hsp90) in all cell types studied compared to untreated controls. However, the levels were found to be decreased compared to those induced by heat shock treatment at



42°C, with the exception of Hsp60. This decrease could have been due to partial denaturation of the cellular content or cell death following an elevated temperature stress, or due to the fact that the proteins were no longer required as the cellular stress had been minimised during the initial stage of the stress response or even due to a potential translocation of the HSPs to the cell surface.

### **3.4.3 Translocation of HSPs.**

Current opinion suggests the appearance of HSPs on the cell surface (in non-permeabilized cells) is limited to human cancer cells and transformed cell lines (Multhoff et al. 1995), and in agreement with this a detectable level of membrane-bound Hsp72 was measured on U937 monocytes. However, this study also revealed appreciable levels of Hsp72 on normal human leukocytes taken from healthy volunteers, both membrane-bound Hsp72 and also membrane-embedded Hsp72; the latter measured with the cm.Hsp70.1 antibody. This study found neutrophils and lymphocytes to express significant levels of membrane-embedded Hsp72, whilst monocytes were found to express significant levels of membrane-bound Hsp72. In addition detectable levels of membrane-bound Hsp27, Hsp60 and Hsp90 were also found on human leukocyte membranes. High-salt concentrations were found to alter HSP membrane-bound expression, a finding which indicates that the proteins were bound to a cell surface component, potentially a cell surface receptor (Thériault et al. 2005; Thériault et al. 2006). However, membrane-embedded Hsp72 expression suggests Hsp72 may be associated with fatty acids within the plasma membrane, previously suggested over two decades ago by Hightower & Guidon in 1989. The data in this chapter is one of the first studies to reveal the localisation of HSPs on the membrane of non-permeabilized healthy whole blood samples.

Cells exposed to the elevated temperature of 42°C for one hour were found to have an increased HSP (Hsp27, Hsp60, Hsp72 and Hsp90) cell surface (membrane-bound or membrane-embedded) expression in a time-dependent and cell specific manner. It was found that an elevated temperature of 42°C increased the cell surface (membrane-bound) expression of Hsp72 on U937 cells. This is similar to the findings of Tani et al. (2009), who found exposure of a murine tumour cell line to 42°C for two hours induced the transportation of a fraction of inducible Hsp72 to the cell surface. The location of Hsp72 at the cell surface is in

agreement with previous studies who suggest the expression of Hsp72 at the cell surface to be restricted to human cancer cells (Multhoff et al. 1995), where it is shown to be associated with cytolytic attack from NK cells and subsequently apoptosis (Botzler et al. 1996; Multhoff et al. 1999; Gehrmann et al. 2003; Kleinjung et al. 2003; Gastpar et al. 2005; Schmitt et al. 2007). Indeed, the cell surface expression of Hsp72 on U937 cells in this study was positively associated with PS externalisation analysed by Annexin V on the flow cytometer after a one hour heat shock at 42°C. The presence of PS on the external membrane is characteristic of cells undergoing apoptosis. Furthermore, Hsp72 expression was also induced in human leukocytes however, its expression varied between the leukocyte subtypes. Consistently, both neutrophils (membrane-embedded) and monocytes (membrane-bound) up-regulated a significant increased level of cell surface Hsp72 compared to lymphocytes. Again, a high-salt concentration altered Hsp72 membrane expression, indicating that the proteins were bound to a cell surface component as well as being membrane-embedded. It has been proposed that the presence of Hsp72 on the cell surface (membrane-bound or embedded) may function to stabilise the membrane during heat shock treatment, controlling its function such as the distribution of integral membrane proteins and help in the recovery process from the effects of elevated temperature stress (Vigh et al. 2007). In contrast, it has also been suggested that the up-regulation of cell surface Hsp72 is directly linked with cells undergoing apoptosis (Poccia et al. 1996; Sapozhnikov et al. 1999; Sapozhnikov et al. 2002; Feng et al. 2003; Gross et al. 2003b; Korbelik et al. 2005). This study demonstrates a positive association between on the onset of apoptosis and cell surface HSP expression. It was revealed that a significant increase in cell surface Hsp72 on neutrophils, lymphocytes and monocytes was positively associated with PS externalisation after a one hour heat shock treatment at 42°C. This is in agreement with Korbelik et al. (2005) who have shown photodynamic therapy to induce the cell surface expression of Hsp72 and Hsp60 in combination with PS during apoptosis in the mouse cell line SCCV11. This up-regulation is of a potential protective nature for the immune system, for the removal of unresponsive or redundant cells as a result of the heat shock, which may lead to problems such as immuno-pathology if they were not removed. Indeed, it has been shown that apoptosis is important for controlling stages in inflammatory and immunological reactions and in the

protection from ROS (Galea-Lauri et al. 1996), of which leukocytes perform an important role. Despite the evidence of a positive association between surface HSPs and apoptosis, the factors controlling the transportation of HSPs to the cell membrane during apoptosis remain unclear. HSPs do not have a consensual secretory signal therefore the mechanism for translocation of HSPs across membranes is debatable. However, several mechanisms of translocation have been proposed which use the non-classical pathway involving HSPs being transported to the cell surface after binding to other proteins or structures. These include, lipid rafts (Triantafilou & Triantafilou, 2002b; Broquet et al. 2003; Chen et al 2005), exosomes (Clayton et al. 2005; Gastpar et al. 2005; Lancaster & Febbraio, 2005), endolysosomes (Mambula & Calderwood, 2006a; Mambula & Calderwood, 2006b), secretory-like granules (Evdonin et al. 2006) and a leaderless secretion involving ABC-like transporters resembling IL- $\beta$  release (Mambula & Calderwood, 2006a; Mambula & Calderwood, 2006b). Thus, it seems the translocation of HSPs from cells is stress and cell type dependent.

A one hour heat shock treatment was also able to stimulate the cell surface (membrane-bound) expression of Hsp27, Hsp60 and Hsp90 expression in human leukocytes, however there was a reduced up-regulation when compared to Hsp72. Neutrophils up-regulated a significant level of cell surface Hsp27, Hsp60 and Hsp90 compared to untreated controls and to lymphocytes. However, interestingly monocytes were shown to exhibit the highest up-regulation of Hsp27 and Hsp60 compared to both neutrophils and lymphocytes. The presence of Hsp27 on the cell surface maybe related to its ability to stabilise actin microfilaments through binding to F-actin to prevent disruption of the cytoskeleton resulting from heat shock (Guay et al. 1997; Rayner et al. 2008). In addition, Hsp60 on the surface of monocytes complements a previous study by Bethke et al. (2002), who found preparations of human Hsp60 applied to human monocytes to activate the innate immune response. Furthermore, the expression of cell surface Hsp27 and Hsp60 may also represent a cellular mechanism for controlling signalling events at the cell membrane (Broquet et al. 2003), which correlates with the function of monocytes in the process of antigen processing and presentation. The surface expression of Hsp27 and Hsp60 was found to positively associate with PS externalisation after the heat shock treatment which agrees with the proposed functions of these HSPs when found on the cell surface.

Similar to the induced expression of Hsp72 on the cell membrane, a high-salt concentration altered HSP membrane expression, indicating that the proteins were bound to a cell surface component after a heat shock treatment.

Finally, it was also shown that cells incubated for three hours at 37°C, following exposure to elevated temperature stress at 42°C, induced the translocation of HSP (Hsp27, Hsp60, Hsp72 and Hsp90) to the cell surface in a time-dependent and cell specific manner. U937 cells were shown to express increased levels of cell surface Hsp72 after three hour incubation at 37°C. Furthermore, human leukocytes including neutrophils, lymphocytes and monocytes were all also found to express increased levels of Hsp72 on their cell surface, membrane-bound or membrane-embedded compared to untreated controls. These results suggest the unusual translocation of Hsp72 from inside human leukocytes to the cell surface during and after the process of heat-induced apoptosis. This suggests HSPs may initially associate with PS on the cytosolic phase of the membrane and translocate within the bilayer by spontaneous ‘flipping’ outside of the cell during the process of apoptosis. Indeed previous experiments have established Hsp72 and Hsc70 to interact with lipid membranes (Arispe & De Maio, 2000; Schilling et al. 2009), displaying a high degree of specificity for the presence of PS, inducing the synthesis of pro-inflammatory cytokines (Asea et al. 2002), followed by rapid incorporation into the lipid bilayer (Arispe et al. 2004). More recently, Vega et al. (2008) have demonstrated Hsp72 inserted into the plasma membrane before release into the extracellular environment in membrane-associated structures from intact stressed cells. The mechanism by which Hsp72 is inserted into the membrane is still not fully understood, however it has been proposed that Hsp72 is capable of assembling into low-order oligomers when the protein is in excess of polypeptide targets when proteins become unfolded after stress (Arispe et al. 2004). Similar to the expression of Hsp72, the cell surface expression of Hsp90 on human leukocytes also increased compared to untreated controls following a period of recovery after heat shock treatment at 42°C. These results are in agreement with previous studies which revealed within the brain, Hsp90, Hsc70 and Hsp60 are found in synaptic membrane fractions isolated by biochemical methods (Bechtold et al. 2000). The results in this chapter suggest the cell surface translocation of HSPs is a consequence of membrane alteration and a result of heat-induced apoptosis, therefore it may be proposed that cell

surface HSPs are an indicator of cellular damage and a mechanism by which cells respond to an intensive heat shock. Another possible function for the translocation of both Hsp72 and Hsp90 to the cell surface after heat shock treatment may be to stabilise the membrane (Vigh et al. 2007) and to help in the recovery process by re-establishing the fluidity and bilayer stability of the plasma membrane (Török et al. 1997; Arispe & De Maio, 2000; Arispe et al. 2004; Vega et al. 2008), restoring membrane functionality and helping cells to overcome the denaturation of trans-membranal transport proteins and surface receptors during stress (Török et al. 1997; Hildebrandt et al. 2002). This will be explored in chapters four and five. Furthermore, the presence of these HSPs on the cell surface after stress may function as a ‘danger signal’ to the immune system, in inflammation or immunity, stimulating an immune response to a subsequent stress. This issue will be explored in chapter six. In contrast, the cell surface expressions of both Hsp27 and Hsp60 were decreased compared to heat shocked levels after a period of recovery at 37°C. The decrease in cell surface levels may indicate a potential release of the proteins into the extracellular milieu, a process which may lead to the production of intracellular HSP in order replenish levels following heat stress. This may be the situation for Hsp60, where following incubation at 37°C for three hours, the whole cell levels of Hsp60 increased.

In conclusion, this chapter suggests Hsp72 is the predominant HSP that is expressed intracellularly under normal physiological conditions (37°C), and induced intracellularly in both cultured (U937 monocytes) and peripheral blood cells (human leukocytes) under conditions of elevated temperature stress (42°C). It also reports the simultaneous translocation of intracellular HSPs, in particular Hsp72 and PS to the cell surface of both cell types in response to elevated temperature stress. The relationship between the progression of apoptosis and/or necrosis, and the increased expression of HSPs either on the cell surface (membrane-bound and membrane-embedded) or whole cell suggests a role for HSPs in apoptotic and necrotic cell death. Future experiments would require mimicking the *in vitro* situation *in vivo* in order to investigate the effects of hyperthermia and HSPs in further detail, thus requiring the determination of the effect of whole body hyperthermia.

## **Chapter 4**

### **The protection of U937 cells and human leukocytes by extracellular Hsp70.**

#### **4.1 Introduction**

Several groups have investigated the conditions which initiate apoptotic or necrotic cell death (Bellman et al. 1996; Buzzard et al. 1998; Creagh et al. 2000; Kim et al. 2000; Milleron & Bratton, 2006), and this has been demonstrated in chapter three, therefore the work in this chapter will focus on methods to directly intervene and protect cells from heat shock induced injury. For many years HSPs were traditionally regarded as intracellular stress proteins that were up-regulated in response to physiological conditions or environmental stresses (Lindquist & Craig, 1988; Morimoto, 1994; Hartl, 1996). HSPs have since been found to be present outside of the cell, in the extracellular milieu (Tytell et al. 1986; Hightower & Guidon, 1989; Pockley et al. 1998; Wright et al. 2000), released from viable (Guzhova et al. 2001; Barreto et al. 2003; Hunter-Lavin et al. 2004a; Bausero et al. 2005; Davies et al. 2006), or necrotic cells (Gallucci et al. 1999; Basu et al. 2000b; Berwin et al. 2001; Saito et al. 2005), and on the plasma membrane (Poccia et al. 1992; Multhoff et al. 1995; Sapozhnikov et al. 1999; Sapozhnikov et al. 2002; Kleinjung et al. 2003; Korbelik et al. 2005; Steiner et al. 2006; Cid et al. 2008; Gehrmann et al. 2008) as demonstrated in chapter three. Depending on their localisation, extracellular HSPs have been reported to elicit a stimulatory effect on the immune response (Asea et al. 2000b; Matzinger, 2002; Campisi & Fleshner, 2003a; Campisi et al. 2003b; Svensson et al. 2006), including cell activation (Guzhova et al. 1998; Gallucci et al. 1999; Asea et al. 2000b; Basu et al. 2000b) and up-regulation and secretion of cytokines (Singh-Jasuja et al. 2000; Campisi et al. 2003b; Svensson et al. 2006), and also provide protection of cells from physiological and environmental stresses, especially those that produce low levels of HSPs (Johnson & Tytell, 1993; Honenou et al. 1996; Guzhova et al. 1998; Fujihara & Nadler, 1999; Guzhova et al. 2001). Guzhova et al. (1998) utilised pure Hsp/Hsc70 isolated from calf muscle and exposed human pro-monocytic U937 cells to TNF- $\alpha$  treatment alone or in combination with Hsp/Hsc70 100  $\mu$ g/mL. They found Hsp/Hsc70 to protect cells

from chemically induced apoptosis or necrosis through binding and uptake of Hsp/Hsc70. Furthermore, Guzhova et al. (2001) revealed glia cells to release Hsp72, which was shown to increase in response to heat shock. Neuroblastoma cells which are sensitive to heat shock were able to take up Hsp72 from media improving their tolerance to heat shock. This suggests extracellular HSPs *in vivo* may modulate vital cellular functions and are important in the protection of cells which are deficient in HSP production, and thus depend on extracellular HSPs for survival.

The mechanism by which extracellular HSPs improve cell viability is at present not fully understood, however it has been demonstrated that the first step of the effects of extracellular HSPs on cells is the binding of HSPs to a receptor on the cell surface. A number of potential receptors for the binding of HSPs to cell surfaces have been identified and include, TLR2 and TLR4 in a CD14 dependent fashion for Hsp72 (Asea et al. 2000b; Vabulas et al. 2002) and Hsp60 (Kol et al. 2000), CD91 for Hsp72, Hsp90 and grp94 (Binder et al. 2000; Basu et al. 2001), CD40 for Hsp72 (Wang et al. 2001; Becker et al. 2002), which upon binding results in activation of DCs (Millar et al. 2003) and LOX-1 for Hsp72 (Delneste et al. 2002; Thériault et al. 2005). In addition to binding to receptors on the cell surface, the second step of the effects of extracellular HSPs on cells is thought to be the rapid internalisation of the HSPs through receptor-mediated endocytosis (Arnold-Schild et al. 1999; Basu et al. 2001; Becker et al. 2002), which demonstrates the existence of specific receptors for HSPs on specific cells (Arnold-Schild et al. 1999). Despite this evidence it is not yet clear which method of binding to the cell surface or uptake of extracellular HSPs is the most important in the extracellular functions of these proteins.

#### **4.1.1 Aims**

The aims of this chapter are to investigate the effects of extracellular bovine Hsp70 or recombinant human Hsp72 on apoptosis, and/or necrosis in response to elevated temperature stress in U937 monocytes and human leukocytes, and to determine possible cell surface interactions by which the HSPs can elicit these effects.

## **4.2 Methods**

All preparations and cell culture experiments were performed using aseptic technique in a class II tissue culture hood.

### **4.2.1 Preparation of cells for experimental treatment.**

Human monocytic U937 cells were prepared as in section 2.3.12. Human leukocytes were isolated and prepared for treatment as described in section 2.3.10.

### **4.2.2 Preparation of HSPs for experimental treatment.**

HSPs used for experiments were: bovine Hsp70 or recombinant human Hsp72. Stock solutions used were 0.5 mg/mL in dH<sub>2</sub>O (Bovine Hsp70), and 0.1 mg/mL in PBS (Human Hsp72).

### **4.2.3 Treatment of cells with bovine Hsp70 or human Hsp72.**

Bovine Hsp70 and human Hsp72 were made-up to the appropriate concentrations of 50, 40, 30, 20, 10, 5 and 0 µg/mL in phenol red-free 10 % RPMI before being applied to cells for 1 hour at 37°C.

### **4.2.4 Time course experiments with bovine Hsp70 or human Hsp72.**

Bovine Hsp70 or human Hsp72 were applied to U937 cells or human leukocytes at a concentration of 10 µg/mL diluted in phenol red-free 10 % RPMI. Cells were then incubated at 37°C for up to 4 hours.

### **4.2.5 Pre-treatment of cells with bovine Hsp70 or human Hsp72.**

Bovine Hsp70 and human Hsp72 were made-up to 10 µg/mL in phenol red-free 10 % RPMI before being applied to cells for 2 hours at 37°C before further treatments.

### **4.2.6 Measurement of surface and internalised HSPs.**

Flow cytometry and fluorescence microscopy were used to measure bovine Hsp70 or human Hsp72 on the cell surface (membrane-bound on non-permeabilized cells) or on/inside (permeabilized cells) U937 cells and human leukocytes (Sections 2.3.20 and 2.3.21).



Cells were incubated with an anti-Hsp72 FITC conjugated antibody for 40 minutes at 4°C in the dark.

- 1) Flow Cytometry: Anti-Hsp72: FITC was applied to cells at a 1:250 dilution (50 µL/sample diluted in antibody wash buffer: DPBS containing 5 % FBS).
- 2) Fluorescence Microscopy: Anti-Hsp72: FITC was applied to cells at a 1:100 dilution (50 µL/sample diluted in DPBS containing 1 % BSA).

#### **4.2.7 Time course experiments with heat shock.**

Three *in vitro* heat treatments were performed using:

- 1) A thermal gradient bar set to 37.7°C–46.2°C for 0-6 hours then transferred to a 37°C incubator with 5 % CO<sub>2</sub> for 1 hour recovery.
- 2) A temperature-controlled stirred water bath set to 37°C or 42°C for 1 hour followed by 1 hour recovery at 37°C.
- 3) A temperature-controlled stirred water bath set to 37°C, 42°C or 44°C for 0-2 hours followed by 1 hour recovery at 37°C.

#### **4.2.8 Determination of apoptosis.**

Apoptosis was measured by the microplate based caspase-3 fluorimetric assay (Section 2.3.16), or by flow cytometry analysis of Annexin V (Section 2.3.15) and caspase-3 (Section 2.3.17).

#### **4.2.9 Determination of necrosis.**

Necrosis was determined by PI staining (Section 2.3.18).

#### **4.2.10 Determination of cell viability and proliferation.**

Cell viability was estimated using a colorimetric assay, CellTiter 96® AQueous One Solution Cell Proliferation assay (Section 2.3.19).

#### **4.2.11 Detection of potential receptors for Hsp70.**

The presence of potential Hsp70 receptors on the cell surface of U937 cells or human leukocytes were tested using two methods (Section 2.3.29):

- 1) Competitive inhibition experiments using: Annexin V for PS analysis (2.5 µL/well diluted in 50 µL of 1X binding buffer) at 37°C or following a one hour heat shock treatment at 42°C. Or using known receptor agonists' malBSA,

fucoidan, or poly (I) (100  $\mu$ L/well of 10  $\mu$ g/mL dilution in antibody wash buffer) at 37°C.

2) Receptor specific antibodies: anti-LOX-1 or anti-SR-A1 (100  $\mu$ L/well of a 1:10 or 1:50 dilution in antibody wash buffer), and secondary R-PE or FITC conjugated IgG antibodies (100  $\mu$ L/well of a 1:10 or 1:50 dilution in antibody wash buffer) at 37°C and following a one hour heat shock treatment at 42°C.

### 4.3 Results

The first part of the study was to determine whether bovine Hsp70 or recombinant human Hsp72 could interact with the cell surface of U937 cells and human leukocytes, be readily internalised and protect these cells from the effects of elevated temperature stress. The following quantitative experiments were designed to detect bovine Hsp70 or human Hsp72 associated with the cell surface or internalised by U937 cells using flow cytometry (Figures 4.1-4.6) and fluorescence microscopy (Figures 4.3-4.5) respectively.

Surface binding and internalisation of HSPs in U937 cells following treatments with extracellular bovine Hsp70 or human Hsp72 are presented in Figures 4.1–4.5 and Table 4.1.

#### 4.3.1 Binding and internalisation of extracellular HSPs in U937 cells.

The detection of surface bound (non-permeabilized cells) or surface/intracellular (permeabilized cells) HSPs in U937 cells ( $3 \times 10^5$  cells/mL) was determined following the incubation of cells with increasing concentrations of extracellular bovine Hsp70 or human Hsp72 (0-50  $\mu\text{g/mL}$ ) for one hour at 37°C (Figure 4.1). As shown in the dose response curves presented in figure 4.1A-B, when U937 cells were incubated with extracellular HSPs whether bovine or human above 5  $\mu\text{g/mL}$ , a significant increase in surface binding ( $P < 0.001$ , Figure 4.1A) and internalisation ( $P < 0.001$ , Figure 4.1B) was observed compared to control. A significant increase of binding, 3-fold ( $P < 0.001$ ) and internalisation, 2-fold ( $P < 0.001$ ) was observed with 10  $\mu\text{g/mL}$  bovine Hsp70 or human Hsp72, after a one hour incubation at 37°C compared to control (Figure 4.1A-B). At concentrations lower than this (5  $\mu\text{g/mL}$ ), a non-significant increase of both surface binding and internalisation was observed with bovine Hsp70 (Figure 4.1A-B), however a significant increase of surface binding was observed with human Hsp72 ( $P < 0.001$ , Figure 4.1A) compared to control cells. Surface binding and internalisation of extracellular HSPs in U937 cells was found to increase in a dose-dependent manner ( $P < 0.001$ ) at 5, 10, and 20  $\mu\text{g/mL}$  bovine Hsp70 or human Hsp72 compared to control (Figure 4.1A-B). Beyond these concentrations (30-50  $\mu\text{g/mL}$ ) a plateau in the surface binding and internalisation of HSPs was observed with human Hsp72, however a slow but progressive decline in the

surface binding of HSPs was observed with bovine Hsp70, a 1-fold decrease ( $P < 0.01$ , Figure 4.1A). Significant correlations were seen between surface-bound human Hsp72 and internalised Hsp72,  $r^2 = 0.9782$  ( $P < 0.001$ ), and between surface-bound bovine Hsp70 and internalised Hsp70,  $r^2 = 0.7785$  ( $P < 0.01$ ) (Figure 4.1A-B). A significant amount of human Hsp72 was found to bind to the cell surface of U937 cells ( $P < 0.001$ ) at concentrations  $> 5 \mu\text{g/mL}$  compared to bovine Hsp70 (Figure 4.1), and to internalise ( $P < 0.001$ ) more readily than bovine Hsp70 at concentrations of  $5\text{--}50 \mu\text{g/mL}$  (Figure 4.1B). These data were confirmed by fluorescence microscopy, where binding of bovine Hsp70 to the U937 cell surface (Figure 4.3) and internalisation (Figure 4.4) within one hour was observed. However, no co-localisation of Hsp70 (green) with the nucleus (blue) in U937 cells was observed, indicating that the extracellularly administered Hsp70 protein must be located in the cytosol.

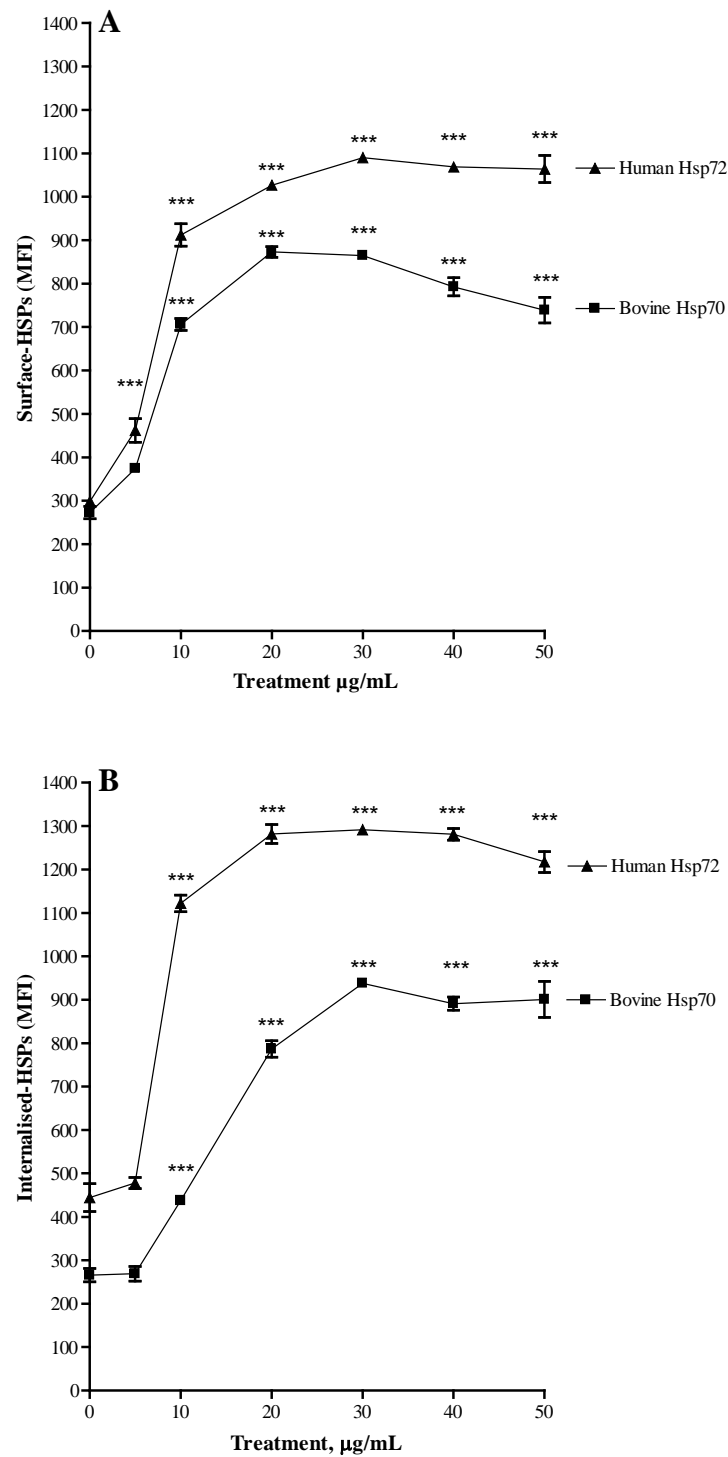
#### **4.3.1.2 Time course of the binding and internalisation of extracellular HSPs in U937 cells.**

A time course of the surface binding and internalisation of bovine Hsp70 or human Hsp72 in U937 cells was then performed over a period of four hours at  $37^\circ\text{C}$ . Following the incubation of U937 cells with extracellular HSPs ( $10 \mu\text{g/mL}$ ) for twenty minutes at  $37^\circ\text{C}$ , a significant 2-fold increase ( $P < 0.05$ ) in the surface binding of human Hsp72 was observed compared to the  $0 \mu\text{g/mL}$  control (Figure 4.2A), however a non-significant increase of internalised human Hsp72 was observed at this time point (Figure 4.4B). In addition, incubation of U937 cells with bovine Hsp70 for twenty minutes observed a non-significant increase of both surface and internalised HSPs compared to control (Figures 4.2A-B). Binding of bovine Hsp70 and human Hsp72 to the U937 cell surface continued to significantly increase over time ( $P < 0.001$ , Figure 4.2A), until two hours where a peak of binding was observed (3-fold increase,  $P < 0.001$ ) compared to control and which was followed by a significant decline in binding, an almost 2-fold decrease ( $P < 0.001$ , Figure 4.2A). In comparison, internalised HSPs significantly increased over time ( $P < 0.001$ , Figure 4.2B), until three hours where a peak of internalisation, an almost 3-fold increase ( $P < 0.001$ , Figure 4.2B) was observed compared control, and which was followed by a significant decline, (a 1-fold decrease,  $P < 0.05$ ) in internal HSPs (Figure 4.2B). Significant

correlations were seen between surface-bound human Hsp72 and internalised Hsp72,  $r^2 = 0.8747$  ( $P < 0.01$ , Figure 4.2A-B), however between surface-bound bovine Hsp70 and internalised Hsp70,  $r^2 = 0.4072$ , there was a non-significant correlation (Figure 4.2A-B). Similarly, a significant amount of human Hsp72 was found to bind to the cell surface of U937 cells ( $P < 0.001$ ) at all time points compared to bovine Hsp70 (Figure 4.2A), and to internalise ( $P < 0.001$ ) more readily than bovine Hsp70 (Figure 4.2B). Consistent with the observations of flow cytometry, fluorescence microscopy (Figures 4.3-4.4) revealed a similar pattern of HSP interaction with U937 cells. Bovine Hsp70 was shown to bind to the cell surface (Figure 4.3) and to internalise (Figure 4.4) in U937 cells after a one hour incubation at 37°C, which increased over time. The results also demonstrated no co-localisation of the extracellularly administered Hsp70 protein (green) with the nucleus (blue) in U937 cells, indicating that the protein must be located in the cytosol.

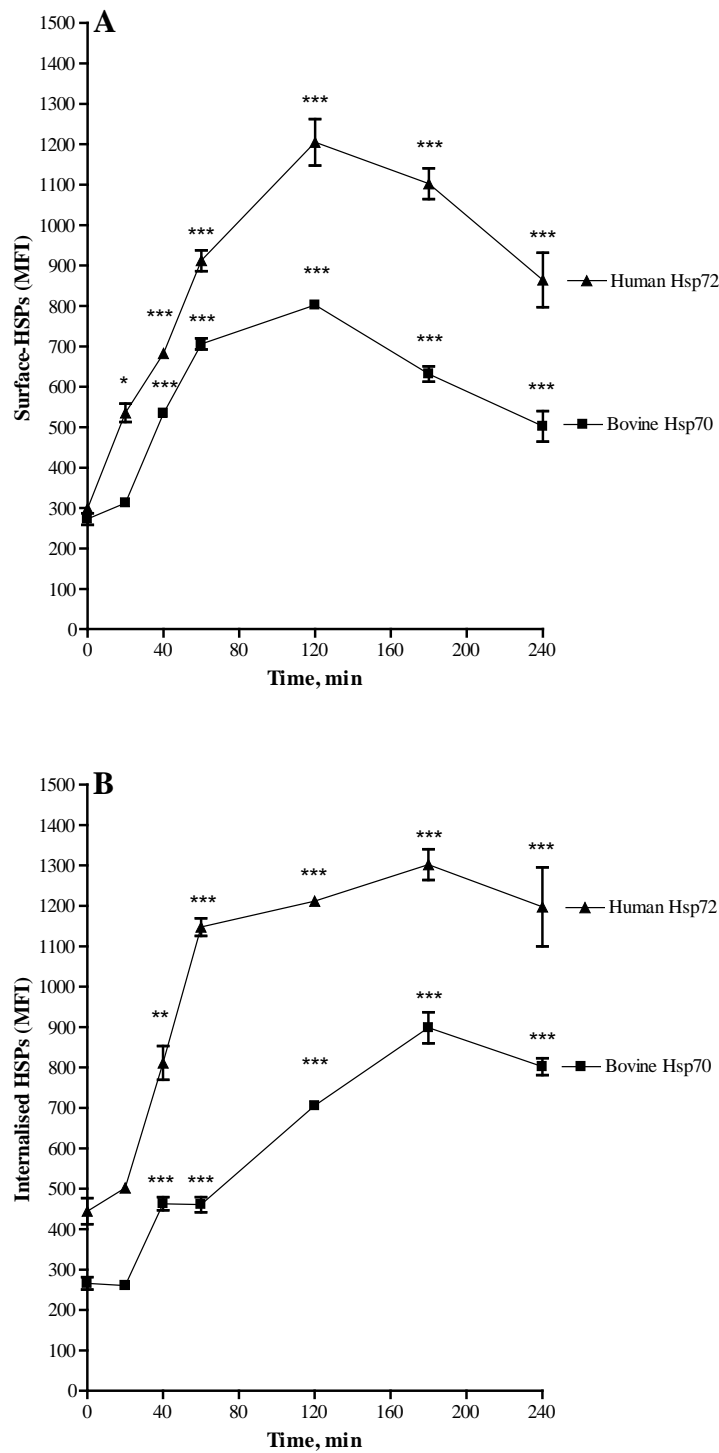
The binding and internalisation of bovine Hsp70 was then determined in U937 cells over a period of four hours at 37°C following a two hour pre-incubation with 10 µg/mL bovine Hsp70. The level of binding of bovine Hsp70 to the U937 cell surface remained unchanged for two hours (Figure 4.5), and then progressively declined with time, to reach an almost 3-fold decrease ( $P < 0.001$ , Figure 4.5) four hours after incubation. In contrast, the level of internalised bovine Hsp70 significantly increased 1-fold ( $P < 0.001$ , Figure 4.5) two hours after incubation. At times less than this (one hour) a non-significant difference from control was observed (Figure 4.5). A plateau of internalisation lasting two hours was seen between two-three hours, which then declined to reach an almost 2-fold decrease ( $P < 0.01$ , Figure 4.5) four hours after incubation.

The addition of extracellular bovine Hsp70 or human Hsp72 (10 µg/mL) to U937 cells for up to four hours did not affect their viability compared to positive controls as determined by MTS assay, caspase-3 activity and PI fluorescence (Table. 4.1).



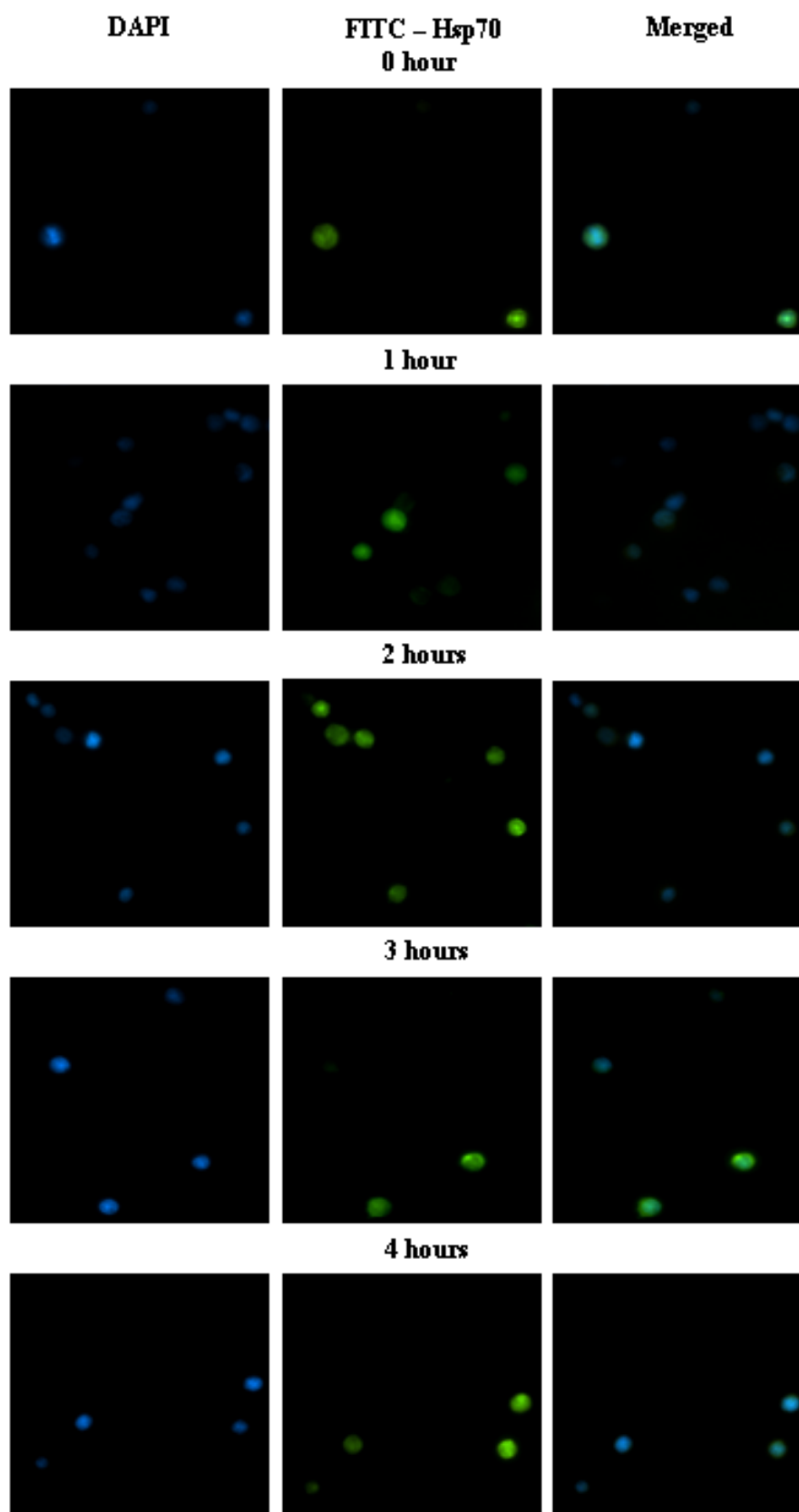
**Figure 4.1: Surface binding (A) and internalisation (B) of HSPs in U937 cells following 1 hour incubation with 0-50 µg/mL bovine Hsp70 or human Hsp72 at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from 0 µg/mL controls between mean concentration points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ). Pearson correlation,  $r^2 = 0.7785$  (Bovine Hsp70),  $r^2 = 0.9782$  (Human Hsp72).



**Figure 4.2: Surface binding (A) and internalisation (B) of HSPs in U937 cells in response to 0-240 minute incubation with 10  $\mu$ g/mL bovine Hsp70 or human Hsp72 at 37°C.**

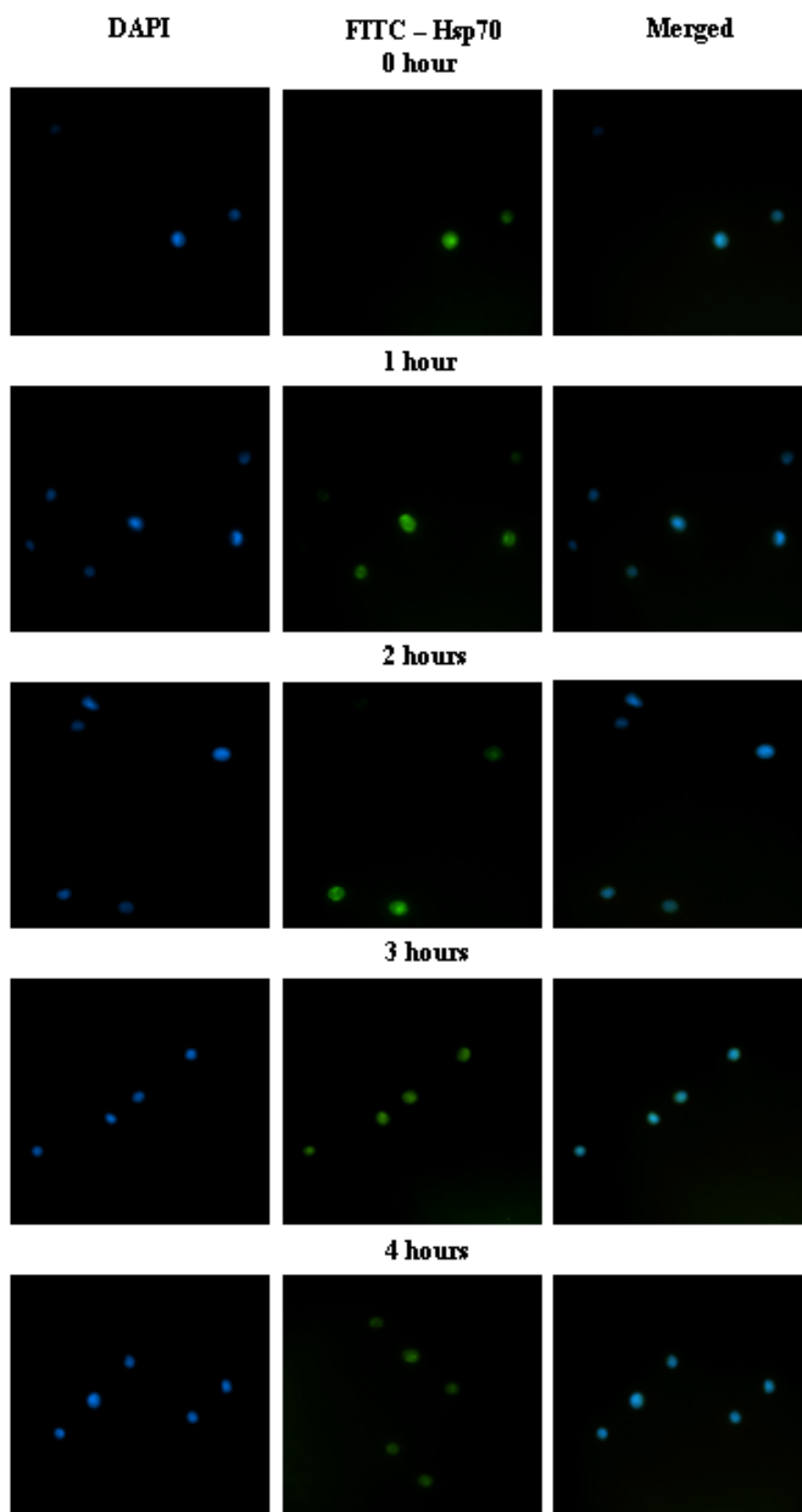
Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean time points compared to time 0 through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001). Pearson correlation,  $r^2 = 0.4072$  (Bovine Hsp70),  $r^2 = 0.8747$  (Human Hsp72).



**Figure 4.3: Surface binding of Hsp70 on U937 cells following a 4 hour time course of incubation with 10  $\mu\text{g}/\text{mL}$  bovine Hsp70 at 37°C.**

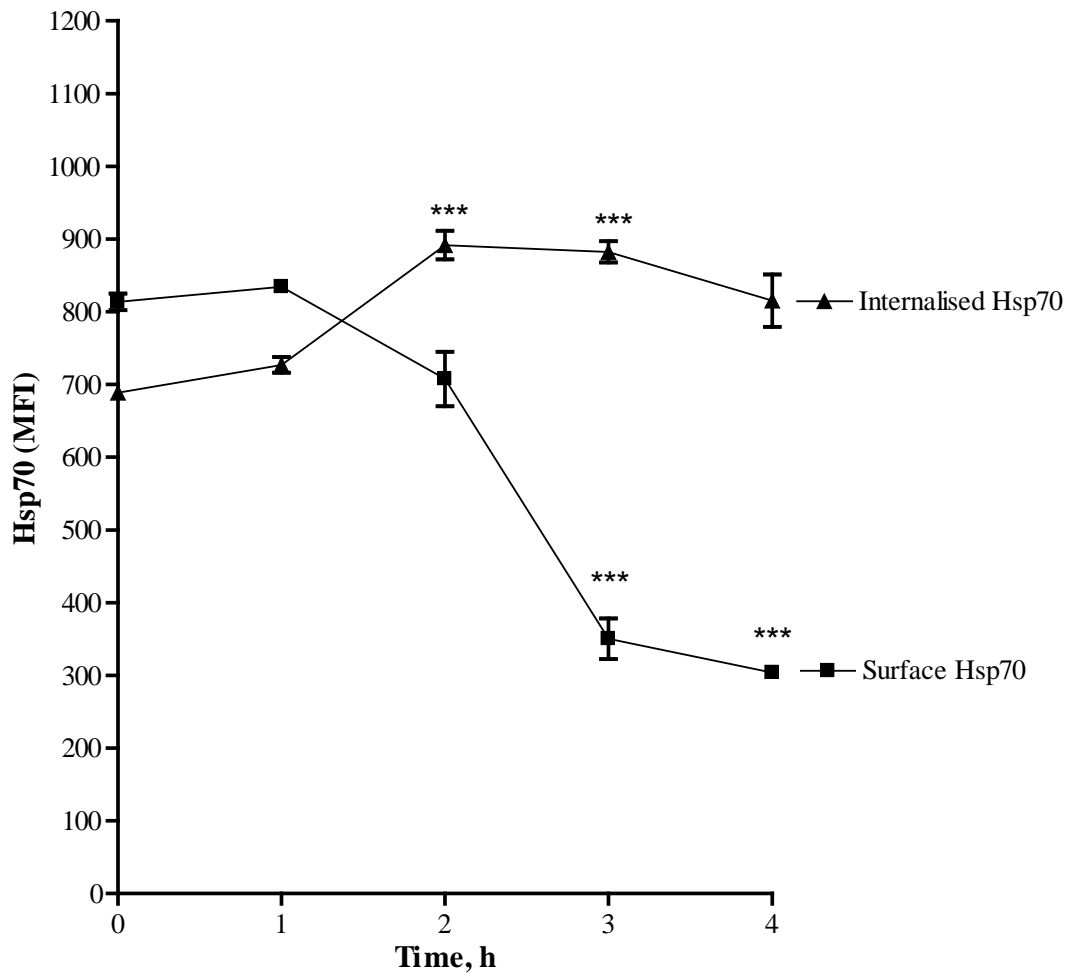
Images were acquired at a magnification of x 40 set up in DAPI (nuclear stain) or FITC (Hsp70 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.





**Figure 4.4: Internalisation of Hsp70 in U937 cells following a 4 hour time course of incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Images were acquired at a magnification of x 40 set up in DAPI (nuclear stain) or FITC (Hsp70 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



**Figure 4.5: Surface binding and internalisation of Hsp70 in U937 cells in response to 0-4 hour recovery at 37°C following a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean time points compared to time 0 through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).

**Table 4.1: Cell viability, caspase-3 and necrosis measurements of U937 cells following incubation with 10 µg/mL bovine Hsp70 for 0-4 hours at 37°C.**

	<b>Time, h</b>	<b>Cell Viability</b> (MTS, AU)	<b>Apoptosis</b> (Caspase-3, RFU)	<b>Necrosis</b> (PI, RFU)
<b>U937 cells</b>	<b>0 (No Treatment)</b>	1.895 ± 0.5	78.00 ± 1.2	12.6 ± 1.5
	<b>1</b>	1.726 ± 0.1	72.00 ± 0.1	15.3 ± 0.2
	<b>2</b>	1.722 ± 0.5	67.00 ± 0.2	13.0 ± 1.2
	<b>3</b>	1.885 ± 0.6	82.00 ± 1.5	14.3 ± 1.5
	<b>4</b>	1.850 ± 1.5	69.00 ± 0.5	12.0 ± 0.6
	<b>Control: Apoptosis</b>	1.435 ± 0.2	2,193 ± 0.1	
	<b>Control: Necrosis</b>	0.370 ± 1.3		322 ± 0.5

Note: Data are presented as SEM, n=3. Caspase-3 positive control = 3 hour heat shock at 40.8°C. PI positive control = 6 hour heat shock at 46.2°C. Seeding density  $3 \times 10^5$ .

### **4.3.2 Binding and internalisation of extracellular HSPs in human leukocytes.**

Before investigating the effect of extracellularly administered bovine Hsp70 or recombinant human Hsp72 preparations on heat shocked human leukocytes, first the ability of the HSPs to interact with the cell surface and to penetrate inside the cells was determined using flow cytometry (Figures 4.6-4.12) and fluorescence microscopy (Figures 4.8-4.10) respectively.

Surface binding and internalisation of HSPs in human leukocytes following treatments with extracellular bovine Hsp70 or human Hsp72 are presented in Figures 4.6-4.11 and Table 4.2-4.4.

The detection of surface bound or internalised HSPs in human leukocytes ( $3 \times 10^5$  cells/mL) was determined following the incubation of cells with increasing concentrations of extracellular bovine Hsp70 or human Hsp72 (0-50  $\mu\text{g/mL}$ ) for one hour at  $37^\circ\text{C}$  (Figures 4.6-4.7). As revealed in the dose response curves presented in figures 4.6-4.7A-B, when human leukocytes were incubated with extracellular HSPs, bovine or human above 5  $\mu\text{g/mL}$ , a significant increase of surface binding ( $P < 0.001$ , Figure 4.6A-B) and internalisation ( $P < 0.001$ , Figure 4.7A-B) was observed compared to control. A significant increase of surface binding was seen on neutrophils, 2-fold ( $P < 0.001$ ); lymphocytes, 2-fold ( $P < 0.001$ ); and also on monocytes, 2-fold ( $P < 0.001$ ) after a one hour incubation with 10  $\mu\text{g/mL}$  HSPs compared to control (Figure 4.6A-B). In addition, a significant increase of internalised HSPs in neutrophils, 2-fold ( $P < 0.001$ ) and monocytes, also a 2-fold increase ( $P < 0.001$ ) was observed with 10  $\mu\text{g/mL}$  bovine Hsp70 or human Hsp72 compared to control (Figure 4.7A-B). In contrast, a non-significant increase of internalised HSPs was seen in lymphocytes with 10  $\mu\text{g/mL}$  bovine Hsp70, however a significant 2-fold increase ( $P < 0.001$ ) was observed with human Hsp72 (Figure 4.7A-B). At concentrations lower than this (5  $\mu\text{g/mL}$ ), a non-significant increase of surface binding was observed on lymphocytes, however a significant increase of surface binding on neutrophils ( $P < 0.05$ ) and monocytes ( $P < 0.001$  bovine Hsp70,  $P < 0.01$  human Hsp70) was observed compared to control (Figure 4.6A-B). Furthermore, a non-significant increase of internalised HSPs was observed in all leukocyte subtypes when incubated with either bovine Hsp70 or human Hsp72 (5  $\mu\text{g/mL}$ ) compared to

control (Figure 4.7A-B). Surface binding and internalisation of extracellular HSPs in human leukocytes was found to increase in a dose-dependent manner ( $P < 0.001$ ) at 5, 10, and 20  $\mu\text{g/mL}$  bovine Hsp70 or human Hsp72 compared to control (Figures 4.6-4.7A-B). Beyond these concentrations (30-50  $\mu\text{g/mL}$ ) a plateau in the surface binding of HSPs to lymphocytes and monocytes was observed, however a significant decline in the surface binding of HSPs on neutrophils was observed with bovine Hsp70, 1-fold decrease ( $P < 0.001$ ), but a non-significant decrease with human Hsp72 (Figure 4.6A-B). Similarly with these concentrations (30-50  $\mu\text{g/mL}$ ), a plateau in the internalisation of HSPs was observed with human Hsp72 in all leukocyte subtypes, however a decline in the internalisation of bovine Hsp70 was observed in both neutrophils, 1-fold decrease and monocytes, also 1-fold decrease yet these differences were found to be non-significant (Figure 4.7A-B). Significant correlations were seen between surface-bound human Hsp72 and internalised Hsp72 in all leukocyte subtypes,  $r^2 = 0.9423$  ( $P < 0.001$ , neutrophils),  $r^2 = 0.9005$  ( $P < 0.01$ , lymphocytes) and  $r^2 = 0.8769$  ( $P < 0.01$ , monocytes) (Table 4.2) and between surface-bound bovine Hsp70 and internalised Hsp70 in neutrophils only,  $r^2 = 0.8659$  ( $P < 0.01$ , Table 4.2). A significant amount of human Hsp72 was found to bind to the cell surface of neutrophils ( $P < 0.001$ ) and monocytes ( $P < 0.05$  to  $P < 0.001$ ) at concentrations of 5-50  $\mu\text{g/mL}$  compared to bovine Hsp70 (Figure 4.6A-B), and to internalise in neutrophils ( $P < 0.001$ ) and monocytes ( $P < 0.001$ ) more readily than bovine Hsp70 at concentrations  $> 5$   $\mu\text{g/mL}$  (Figure 4.7A-B). In both cases there was no significant difference between human Hsp72 and bovine Hsp70 on the cell surface or internalised in lymphocytes (Figures 4.6-4.7A-B). Between the leukocyte subtypes neutrophils were found to bind a significantly greater amount of extracellular HSPs compared to both lymphocytes ( $P < 0.001$ ) and monocytes ( $P < 0.001$ , Figure 4.6A-B), and in contrast to surface binding neutrophils and monocytes internalised a significantly greater amount of extracellular HSPs compared to lymphocytes ( $P < 0.001$ , Figure 4.7A-B). These data were confirmed by fluorescence microscopy, where binding of bovine Hsp70 to the leukocytes surface (Figure 4.9), and internalisation (Figure 4.11) within one hour was observed. However, no co-localisation of Hsp70 (green) with the nucleus (blue) in human leukocytes was observed, indicating that the extracellularly administered Hsp70 protein must be located in the cytosol.

#### **4.3.2.1 Time course of the binding and internalisation of extracellular HSPs in human leukocytes.**

A time course of the surface binding and internalisation of bovine Hsp70 or human Hsp72 in human leukocytes was then performed over a period of four hours at 37°C. Following the incubation of human leukocytes with extracellular HSPs (10 µg/mL) for forty minutes, a significant increase ( $P<0.001$ ) in the surface binding of human Hsp72 was observed compared to control in all leukocyte subtypes (Figure 4.8B), and a significant increase was also seen with bovine Hsp70 on both neutrophils ( $P<0.001$ ) and monocytes ( $P<0.001$ ), however on lymphocytes there was no significant difference from control (Figure 4.8A). In addition, a significant increase ( $P<0.05$  to  $P<0.01$ ) of internalised human Hsp72 was observed after a forty minute incubation compared to control in all leukocytes subtypes (Figure 4.10B), however internalisation of bovine Hsp70 was not found to be significant at forty minutes in both neutrophils and lymphocytes, with the exception of monocytes compared to control ( $P<0.01$ , Figure 4.10A). At incubation times less than this (twenty minutes), a non-significant increase of both surface binding (Figure 4.8A-B) and internalisation (Figure 4.10A-B) was observed in all leukocyte subtypes compared to control.

Binding of bovine Hsp70 and human Hsp72 to the cell surface of human leukocytes continued to significantly increase over time ( $P<0.001$ , Figure 4.8A-B), until one hour with human Hsp72 where a 2-fold increase ( $P<0.001$ ) on neutrophils, a 2-fold increase ( $P<0.001$ ) on lymphocytes and an almost 3-fold increase ( $P<0.001$ ) on monocytes was observed compared to control. At incubation times greater than this a plateau of surface binding was observed in all leukocyte subtypes (Figure 4.8B). Binding of bovine Hsp70 to human leukocytes in contrast continued to significantly increase ( $P<0.001$ ) up to two hours, where a 2-fold increase ( $P<0.001$ ) on neutrophils, an almost 2-fold increase ( $P<0.001$ ) on lymphocytes and an almost 2-fold increase ( $P<0.001$ ) on monocytes was observed compared to control. At incubation times greater than this, a plateau of surface binding was observed on lymphocytes and monocytes but a significant decline (1-fold decrease,  $P<0.05$ ) was seen on neutrophils (Figure 4.8A). In comparison, internalisation of HSPs continued to significantly increase over time ( $P<0.001$ , Figure 4.10A-B), until two hours with human Hsp72 where an almost 2-fold increase ( $P<0.001$ ) in neutrophils, a 2-fold increase ( $P<0.001$ ) in

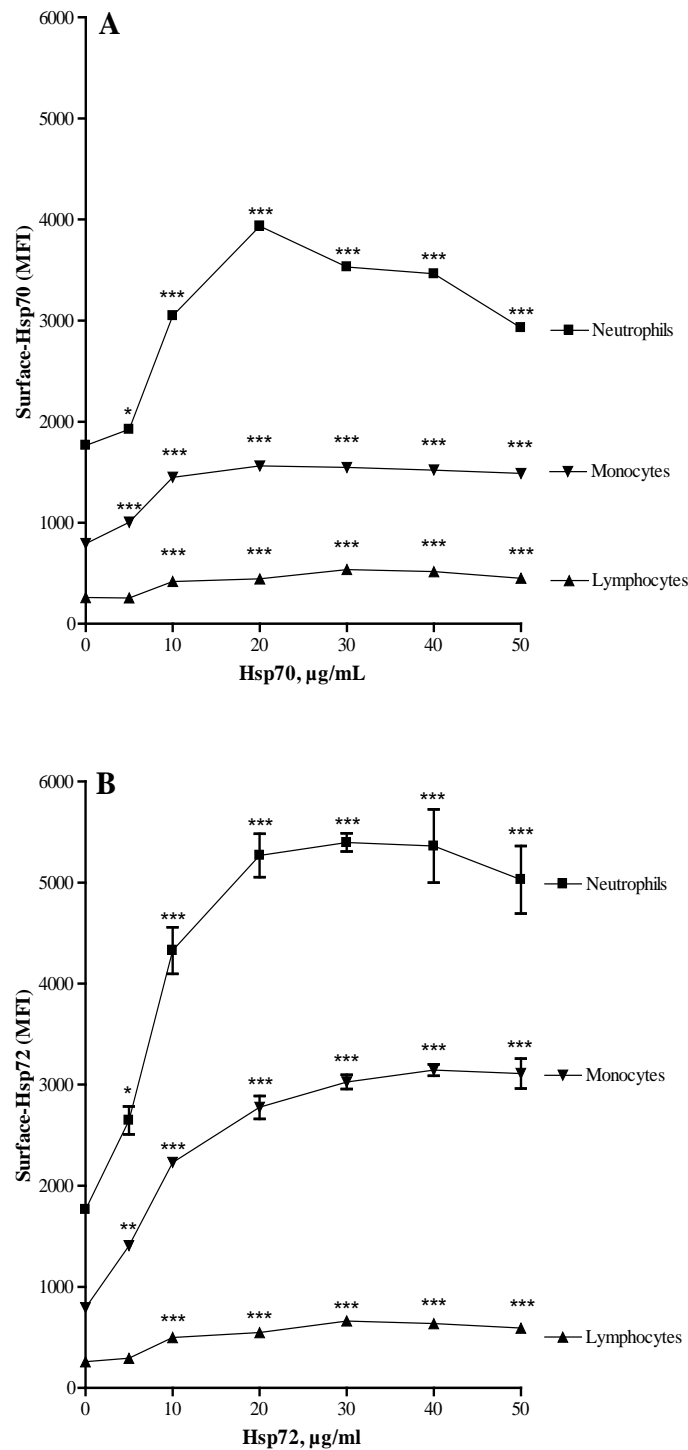
lymphocytes and an almost 3-fold increase ( $P<0.001$ ) in monocytes was observed compared control, and which was followed by a significant decline (a 1-fold decrease,  $P<0.01$ ) in internal HSPs in both neutrophils and lymphocytes but a non-significant decrease in monocytes (Figure 4.10B). Furthermore, internalisation of HSPs in human leukocytes using bovine Hsp70 also significantly increased over time ( $P<0.001$ ), until two hours where an almost 2-fold increase ( $P<0.001$ ) in neutrophils, an almost 2-fold increase ( $P<0.001$ ) in lymphocytes and a 2-fold increase ( $P<0.001$ ) in monocytes was observed compared control, and which was followed by a plateau of internalisation (Figure 4.10A).

Significant correlations were seen between surface-bound human Hsp72 and internalised Hsp72 in all leukocyte subtypes,  $r^2 = 0.778$  ( $P<0.001$ , neutrophils),  $r^2 = 0.5993$  ( $P<0.01$ , lymphocytes) and  $r^2 = 0.7912$  ( $P<0.01$ , monocytes) (Table 4.3) and between surface-bound bovine Hsp70 and internalised Hsp70 in all leukocyte subtypes,  $r^2 = 0.8318$  ( $P<0.01$ , neutrophils),  $r^2 = 0.9445$  ( $P<0.001$ , lymphocytes) and  $r^2 = 0.9657$  ( $P<0.001$ , monocytes) (Table 4.3). Similarly, a significant amount of human Hsp72 was found to bind to the cell surface of neutrophils ( $P<0.001$ ) and monocytes ( $P<0.001$ ) at times above twenty minutes compared to bovine Hsp70 (Figure 4.8A-B), and there was no significant difference between the HSPs on lymphocytes (Figure 4.8A-B). A significant amount of human Hsp72 was also found to internalise in neutrophils ( $P<0.001$ ), lymphocytes ( $P<0.05$ ) and monocytes ( $P<0.001$ ) more readily than bovine Hsp70 at times above forty minutes (Figure 4.10A-B). Again, between the leukocyte subtypes neutrophils were found to bind a significantly greater amount of extracellular HSPs compared to both lymphocytes ( $P<0.001$ ) and monocytes ( $P<0.001$ , Figure 4.8A-B), and in contrast, neutrophils and monocytes internalised a significantly greater amount of extracellular HSPs compared to lymphocytes ( $P<0.001$ , Figure 4.10A-B). Consistent with the observations of flow cytometry, fluorescence microscopy (Figures 4.9-4.11) revealed a similar pattern of HSP interaction with human leukocytes. Bovine Hsp70 was shown to bind to the cell surface (Figure 4.9) and internalise (Figure 4.11) in human leukocytes after a one hour incubation at 37°C, which increased over time. The results also demonstrated no co-localisation of the extracellularly administered

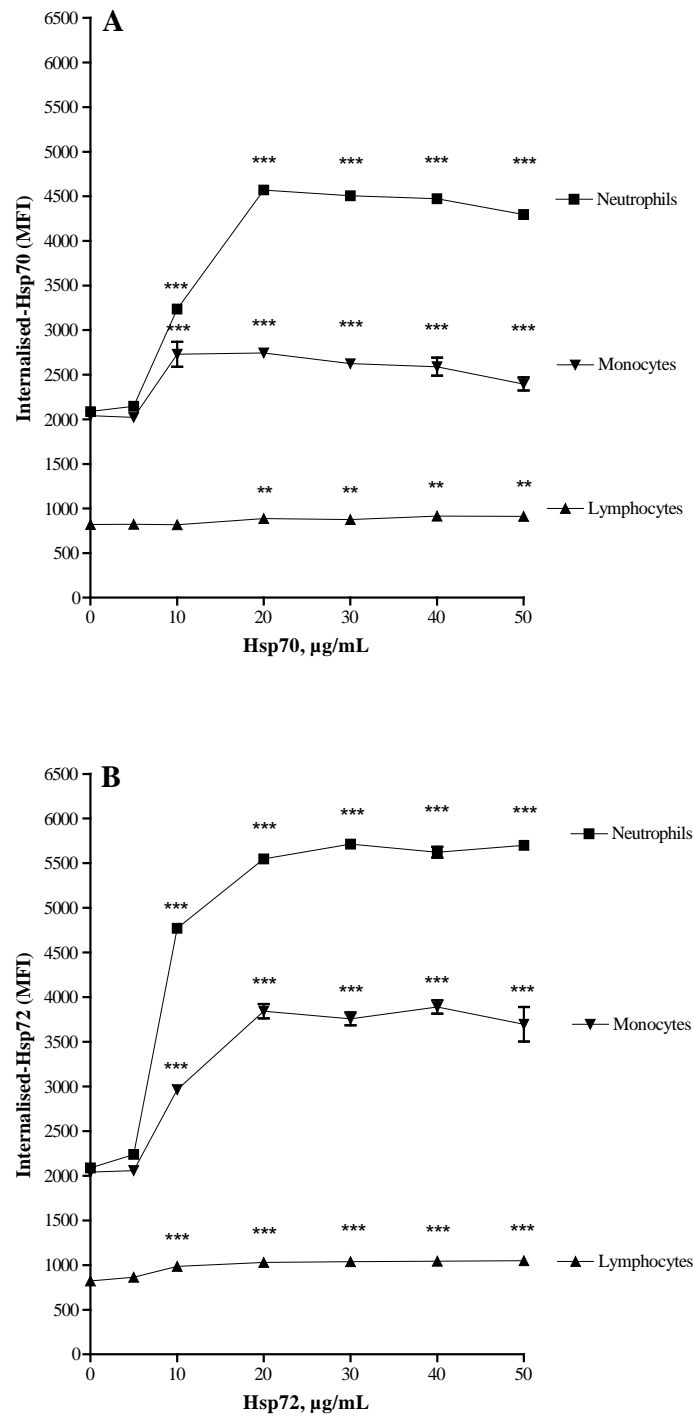
Hsp70 protein (green) with the nucleus (blue) in human leukocytes, indicating that the Hsp70 protein must be located in the cytosol.

The addition of extracellular bovine Hsp70 or human Hsp72 (10 µg/mL) to human leukocytes for up to four hours did not affect their viability compared to positive controls as determined by Annexin V, caspase-3 activity and PI fluorescence (Table. 4.4).





**Figure 4.6: Surface binding of HSPs on human leukocytes following 1 hour incubation with 0-50 µg/mL bovine Hsp70 (A) or human Hsp72 (B) at 37°C.** Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 0 µg/mL controls between mean concentration points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001).

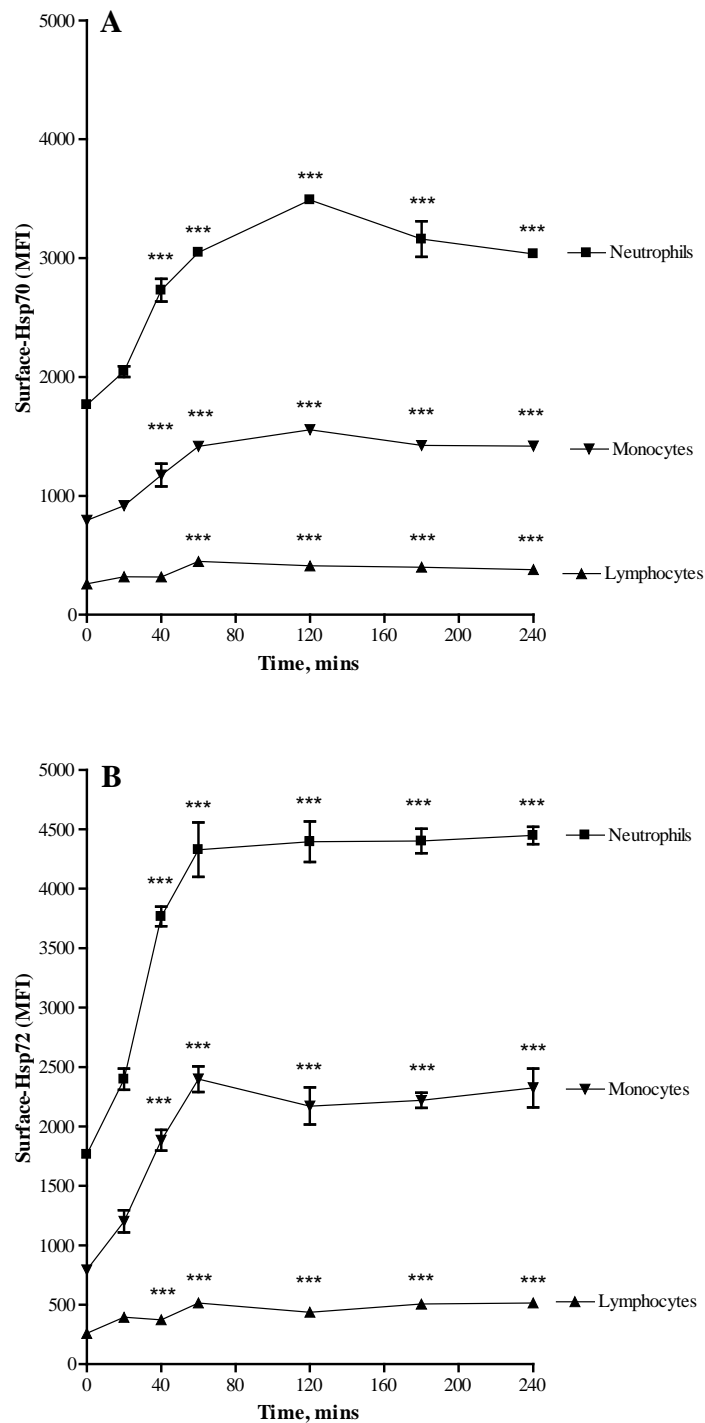


**Figure 4.7: Internalisation of HSPs in human leukocytes following 1 hour incubation with 0-50 µg/mL bovine Hsp70 (A) or human Hsp72 (B) at 37°C.** Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 0 µg/mL controls compared to mean concentration points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.01), \*\*\* (P<0.001).

**Table 4.2: Correlation between the surface binding and internalisation of HSPs in human leukocytes following 1 hour incubation with 0-50 µg/mL bovine Hsp70 or human Hsp72 at 37°C.**

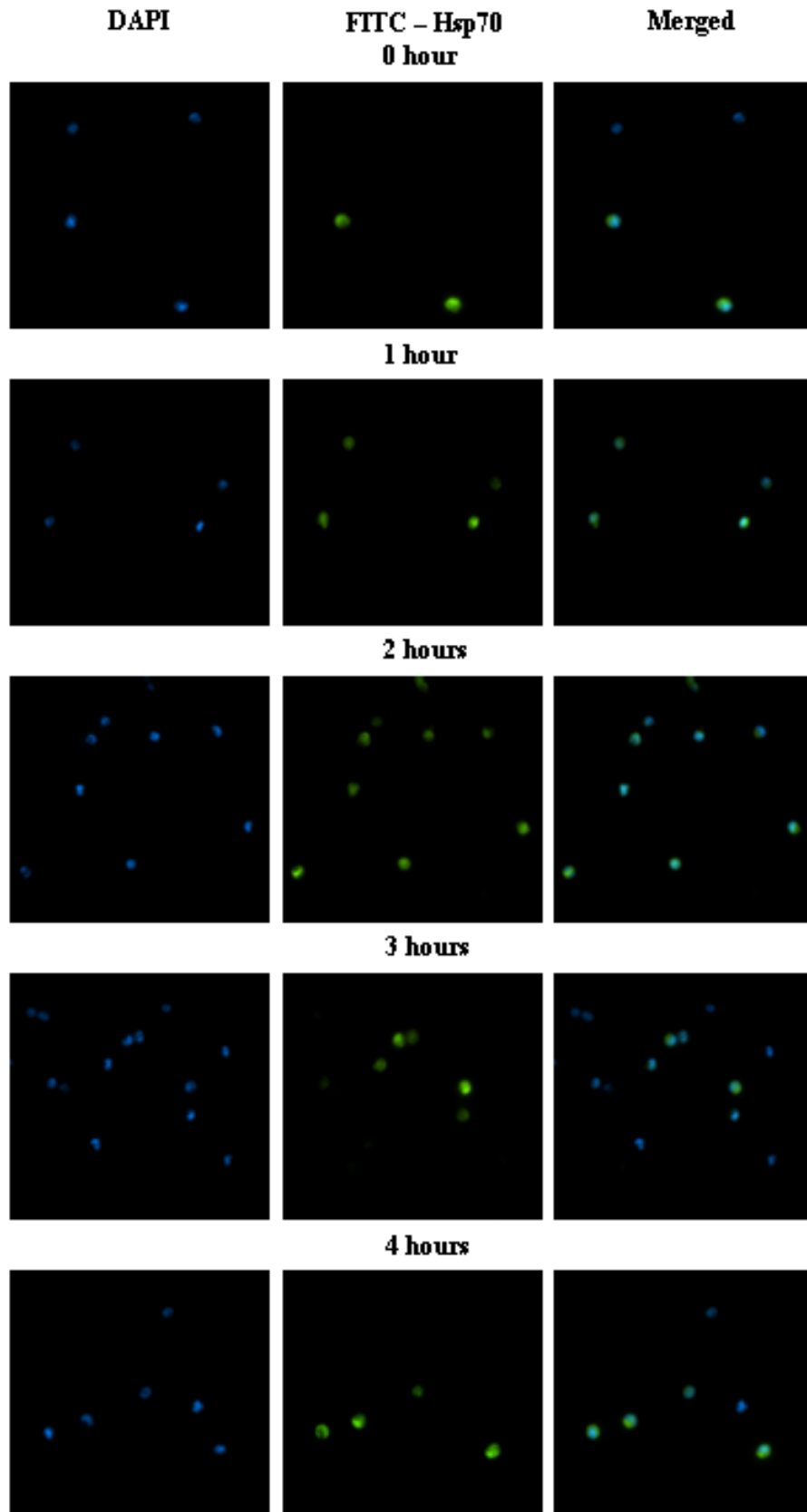
Significance shown following Pearson correlation: \*\* (P<0.01), \*\*\* (P<0.001). Data are representative of three independent experiments.

	<b>Human Hsp72: Internal</b>	
<b>Human Hsp72: Surface</b>	Neutrophils	0.9423 <sup>***</sup>
	Lymphocytes	0.9005 <sup>**</sup>
	Monocytes	0.8769 <sup>**</sup>
	<b>Bovine Hsp70: Internal</b>	
<b>Bovine Hsp70: Surface</b>	Neutrophils	0.8659 <sup>**</sup>
	Lymphocytes	0.0765
	Monocytes	0.1391



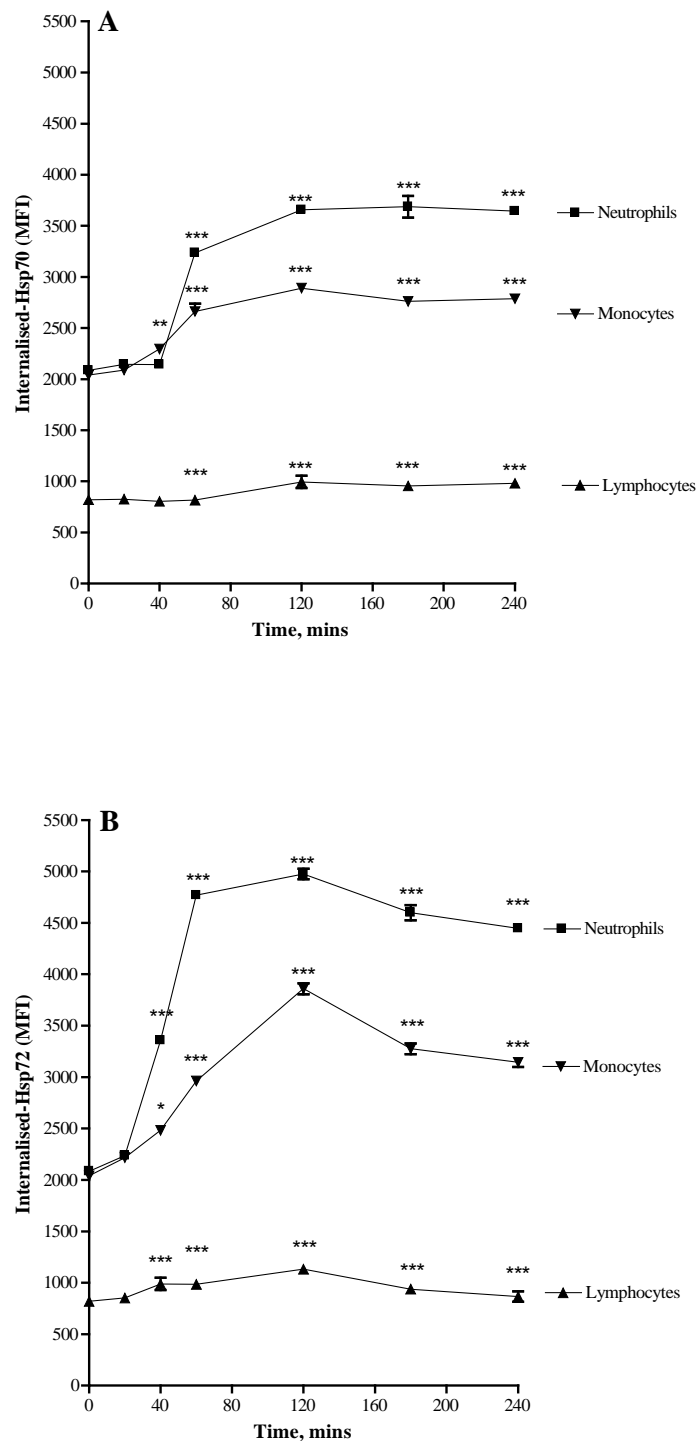
**Figure 4.8: Surface binding of HSPs on human leukocytes in response to 0-240 minute incubation with 10 µg/mL bovine Hsp70 (A) or human Hsp72 (B) at 37°C.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between time points compared to time 0 through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).



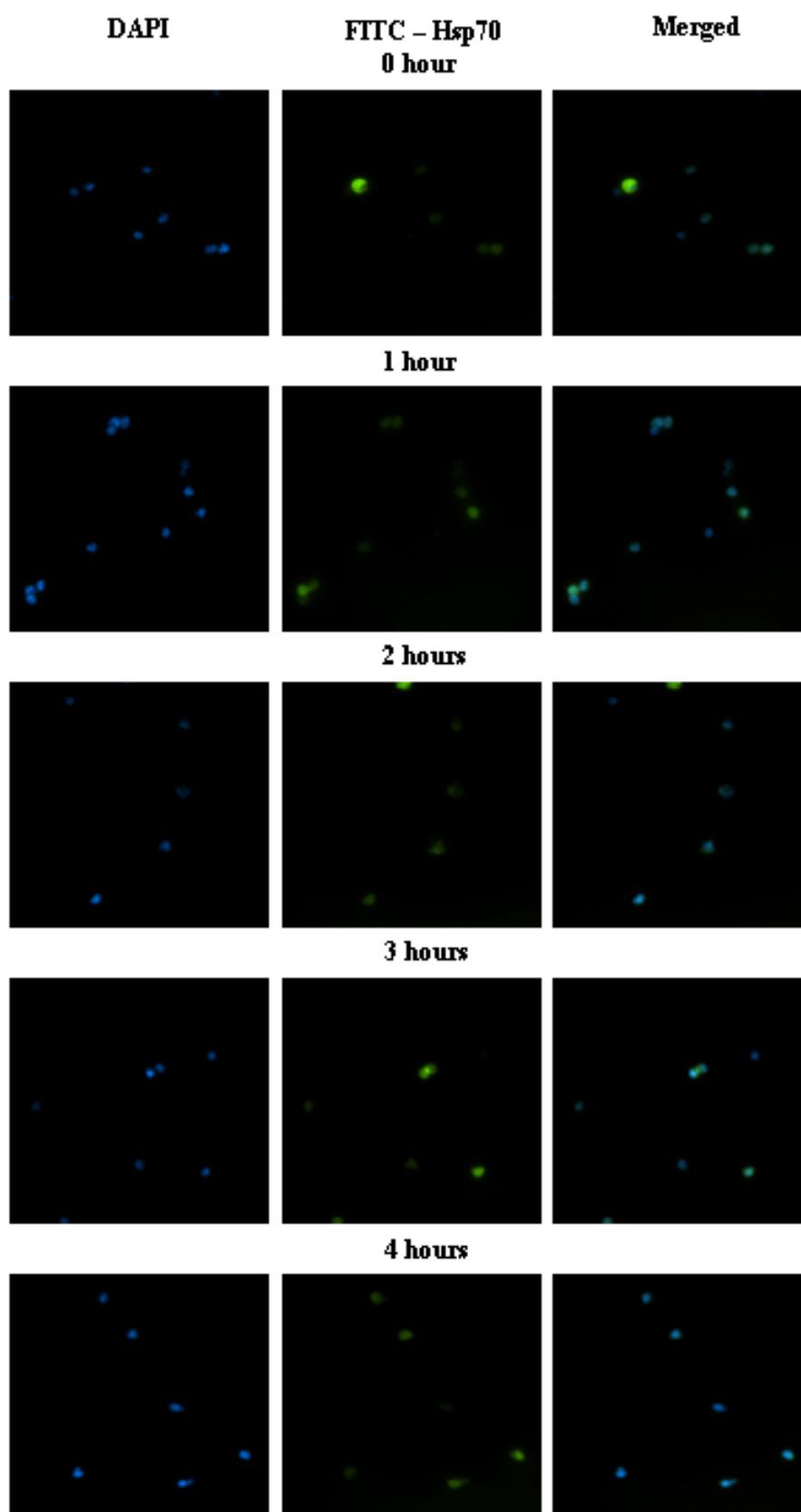
**Figure 4.9: Surface binding of Hsp70 on human leukocytes following a 4 hour time course of incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Images were acquired at a magnification of x 40 set up in DAPI (nuclear stain) or FITC (Hsp70 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



**Figure 4.10: Internalisation of HSPs in human leukocytes in response to 0-240 minute incubation with 10 µg/mL bovine Hsp70 (A) or human Hsp72 (B) at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean time points compared to time 0 through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



**Figure 4.11: Internalisation of Hsp70 in human leukocytes following a 4 hour time course of incubation with 10  $\mu\text{g}/\text{mL}$  bovine Hsp70 at 37°C.**

Images were acquired at a magnification of x 40 set up in DAPI (nuclear stain) or FITC (Hsp70 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.

**Table 4.3: Correlation between the surface binding and internalisation of HSPs in human leukocytes in response to 0-4 hour incubation with 10 µg/mL bovine Hsp70 or human Hsp72 at 37°C.**

Significance shown following Pearson correlation: \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001). Data are representative of three independent experiments.

	<b>Human Hsp72: Internal</b>	
<b>Human Hsp72: Surface</b>	Neutrophils	0.7781**
	Lymphocytes	0.5993*
	Monocytes	0.7912**
	<b>Bovine Hsp70: Internal</b>	
<b>Bovine Hsp70: Surface</b>	Neutrophils	0.8318**
	Lymphocytes	0.9445***
	Monocytes	0.9657***



**Table 4.4: Early apoptosis, caspase-3 and necrosis measurements of human leukocytes following incubation with 10 µg/mL bovine Hsp70 for 0-4 hours at 37°C.**

	Time, h	Early Apoptosis (Annexin V, %)	Apoptosis (Caspase3, %)	Necrosis (PI, %)
<b>Neutrophils</b>	<b>0</b>	0.00 ± 0.5	0.10 ± 0.0	0.40 ± 0.7
	<b>1</b>	0.30 ± 3.1	0.10 ± 0.5	0.25 ± 0.5
	<b>2</b>	0.65 ± 0.5	0.20 ± 0.1	0.80 ± 0.3
	<b>3</b>	0.40 ± 1.5	0.25 ± 0.2	0.45 ± 0.1
	<b>4</b>	0.30 ± 2.5	0.40 ± 0.0	0.35 ± 0.2
	<b>Control: Apoptosis</b>	90.1 ± 2.3	54.6 ± 1.1	
	<b>Control: Necrosis</b>			88.9 ± 0.7
<b>Lymphocytes</b>	<b>0</b>	0.00 ± 0.4	0.00 ± 0.7	0.15 ± 0.5
	<b>1</b>	0.00 ± 1.9	0.35 ± 0.1	0.10 ± 0.0
	<b>2</b>	0.00 ± 1.6	0.10 ± 1.2	0.15 ± 0.5
	<b>3</b>	0.00 ± 1.0	0.00 ± 0.0	0.05 ± 0.5
	<b>4</b>	0.00 ± 1.5	0.15 ± 0.1	0.25 ± 0.5
	<b>Control: Apoptosis</b>	28.4 ± 0.5	33.0 ± 0.1	
	<b>Control: Necrosis</b>			91.2 ± 0.55
<b>Monocytes</b>	<b>0</b>	0.00 ± 1.2	0.00 ± 1.5	0.00 ± 0.0
	<b>1</b>	0.15 ± 0.1	0.30 ± 0.2	0.20 ± 0.0
	<b>2</b>	0.00 ± 0.0	0.10 ± 0.2	0.15 ± 0.5
	<b>3</b>	0.00 ± 0.9	0.00 ± 0.0	0.10 ± 0.5
	<b>4</b>	0.10 ± 0.5	0.00 ± 0.0	0.05 ± 0.5
	<b>Control: Apoptosis</b>	51.7 ± 0.5	42.0 ± 1.5	
	<b>Control: Necrosis</b>			97.9 ± 1.3

Note: Data are presented as SEM, n=3. Annexin V and Caspase-3 positive control = 2 hour heat shock at 42.7°C. PI positive control = 4 hour heat shock at 44.4°C. Seeding density 3 x 10<sup>5</sup>.

### **4.3.3 Extracellular HSPs inhibit the effects of elevated temperature stress in U937 cells.**

In the previous chapter (chapter three) it was established that cells in culture, U937 cells and primary human leukocytes respond to elevated temperature stress, an environmental stressor known to elicit cellular damage and induce cell death *via* apoptosis or necrosis. In this part of the study it was investigated whether extracellular bovine Hsp70 could promote U937 cell survival under these conditions. In the following experiments, U937 cells were exposed to heat shock alone (37.7°C-46.2°C) or in combination with bovine Hsp70 (10 µg/mL). Apoptosis and necrosis were then quantitatively determined using plate-based assays measuring viability, caspase-3 and PI (Figure 4.12) or by flow cytometry detecting Annexin V binding (Figure 4.13).

U937 cell viability, apoptosis and necrosis following treatments with extracellular bovine Hsp70 and elevated temperatures are presented in Figures 4.12-4.13. Surface binding and internalisation of extracellular bovine Hsp70 in U937 cells following treatments with elevated temperatures are presented in Figure 4.14.

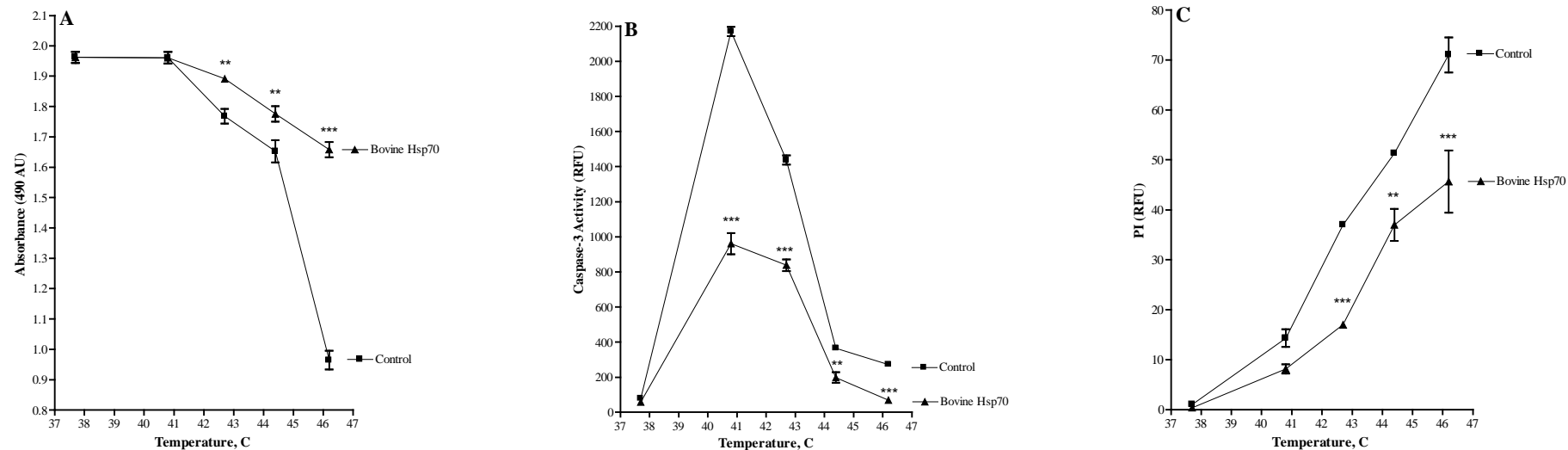
U937 cells ( $3 \times 10^5$  cells/mL) were incubated at temperatures of 37.7°C–46.2°C for up to three hours, followed by one hour recovery at 37°C. As shown in the temperature response curve in figure 4.12, when U937 cells were incubated at temperatures of 40.8°C and above, cell viability was significantly reduced ( $P < 0.01$  to  $P < 0.001$ , Figure 4.12A), as revealed by an increase in the number of apoptotic ( $P < 0.001$ , Figure 4.12B), and necrotic U937 cells ( $P < 0.01$  to  $P < 0.001$ , Figure 4.12C) compared to the 37°C control. The addition of bovine Hsp70 (10 µg/mL) for up to two hours at 37°C prior to heat shock treatment led to a significant, an almost 2-fold increase of cell viability ( $P < 0.001$ , Figure 4.12A) compared to cells treated with heat shock alone. Furthermore, extracellular bovine Hsp70 also significantly prevented (2-fold decrease,  $P < 0.001$ ) apoptosis (Figure 4.12B) and necrosis of U937 cells (an almost 2-fold decrease,  $P < 0.001$ ), compared to cells treated with heat shock alone at all elevated temperatures studied (Figure 4.12C).

Similarly, treatment of U937 cells with extracellular bovine Hsp70 (10 µg/mL) for up to two hours at 37°C prior to incubation at the prolonged exposure time of six hours with the elevated temperatures of 37.7°C–46.2°C, also significantly prevented apoptosis ( $P < 0.05$  to  $P < 0.001$ , Figure 4.13A) and necrosis ( $P < 0.01$  to  $P < 0.001$ , Figure 4.13B) of U937 cells compared to cells treated with heat shock alone (Figure 4.13A-B). Treatment of cells with the physiological temperature of 40.8°C after incubation with bovine Hsp70 (10 µg/mL) resulted in significantly less (72 % to 39 %) ( $P < 0.001$ , Figure 4.13A) apoptotic U937 cells compared to cells treated with heat shock alone. At temperatures above 40.8°C there were also significantly less apoptotic U937 cells compared to cells treated with heat shock alone. A pre-treatment of U937 cells with bovine Hsp70 (10 µg/mL) significantly prevented apoptosis at 42.7°C (59 % to 30 %,  $P < 0.001$ ), 44.4°C (32 % to 12 %,  $P < 0.001$ ), and at 46.2°C (13 % to 5 %,  $P < 0.05$ ), compared to cells exposed to heat shock alone (Figure 4.13A). Previously determined in chapter 3, as the incubation of U937 cells with elevated temperature increased, a decline in apoptotic cell death was observed with a concurrent significant increase in the percentage of necrotic U937 cells compared to the 37°C control ( $P < 0.01$  to  $P < 0.001$ , Figure 4.13B). However, incubation of U937 cells with bovine Hsp70 (10 µg/mL) prior to exposure with elevated temperatures was found to significantly prevent necrosis of U937 cells at temperatures above 40.8°C ( $P < 0.01$  to  $P < 0.001$ , Figure 4.13B). At temperatures below this (40.8°C) there was no significant difference between the percentages of necrotic U937 cells pre-incubated with bovine Hsp70 and those exposed to heat shock alone. There were significantly less necrotic U937 cells at 42.7°C (25 % to 15 %,  $P < 0.01$ ), 44.4°C (42 % to 31 %,  $P < 0.01$ ), and at 46.2°C (90 % to 47 %,  $P < 0.001$ ), compared cells exposed to heat shock alone (Figure 4.13A). Although not all of the temperatures used in these two experiments were physiologically relevant they were used simply to demonstrate protection by extracellular bovine Hsp70.

#### **4.3.3.1 Binding and internalisation of extracellular HSPs following elevated temperature stress in U937 cells.**

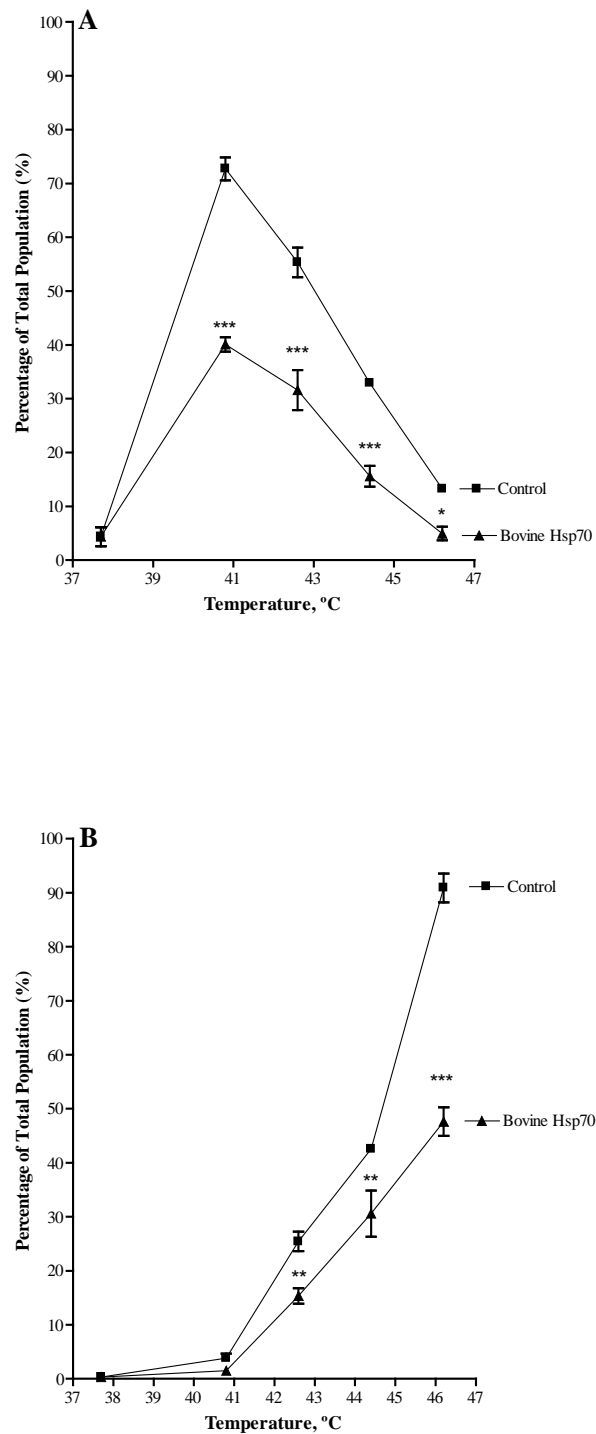
The binding and internalisation of extracellular bovine Hsp70 (10 µg/mL) in U937 cells pre-incubated for two hours at 37°C and following exposure to elevated temperatures (37.7°C–46.2°C) for up to three hours was investigated. A

significant increase of surface binding ( $P<0.05$  to  $P<0.001$ , Figure 4.14) and internalisation ( $P<0.001$ , Figure 4.14) of bovine Hsp70 was observed compared to control at all temperatures studied, with the exception of internalised Hsp70 at 44.4°C and 46.2°C, where a 1-fold decrease ( $P<0.01$ ) was observed, however levels did not reduce to equal those of the control (Figure. 4.14). Binding increased 1-fold ( $P<0.05$ ) at 40.8°C, 1-fold ( $P<0.001$ ) at 42.7°C, 2-fold ( $P<0.001$ ) at 44.4°C and 3-fold ( $P<0.001$ ) at 46.2°C compared to control cells (Figure 4.14). In addition, internalised bovine Hsp70 increased almost 2-fold ( $P<0.001$ ) at 40.8°C and 2-fold ( $P<0.001$ ) at 42.7°C compared to control cells. At temperatures of 42.7°C and below, a significantly ( $P<0.01$  to  $P<0.001$ ) greater amount of bovine Hsp70 was found in U937 cells compared to surface binding (Figure 4.14), and in contrast at temperatures of 44.4°C and above, a significantly ( $P<0.001$ ) greater amount of bovine Hsp70 was detected on the cell surface compared to internal levels (Figure 4.14).



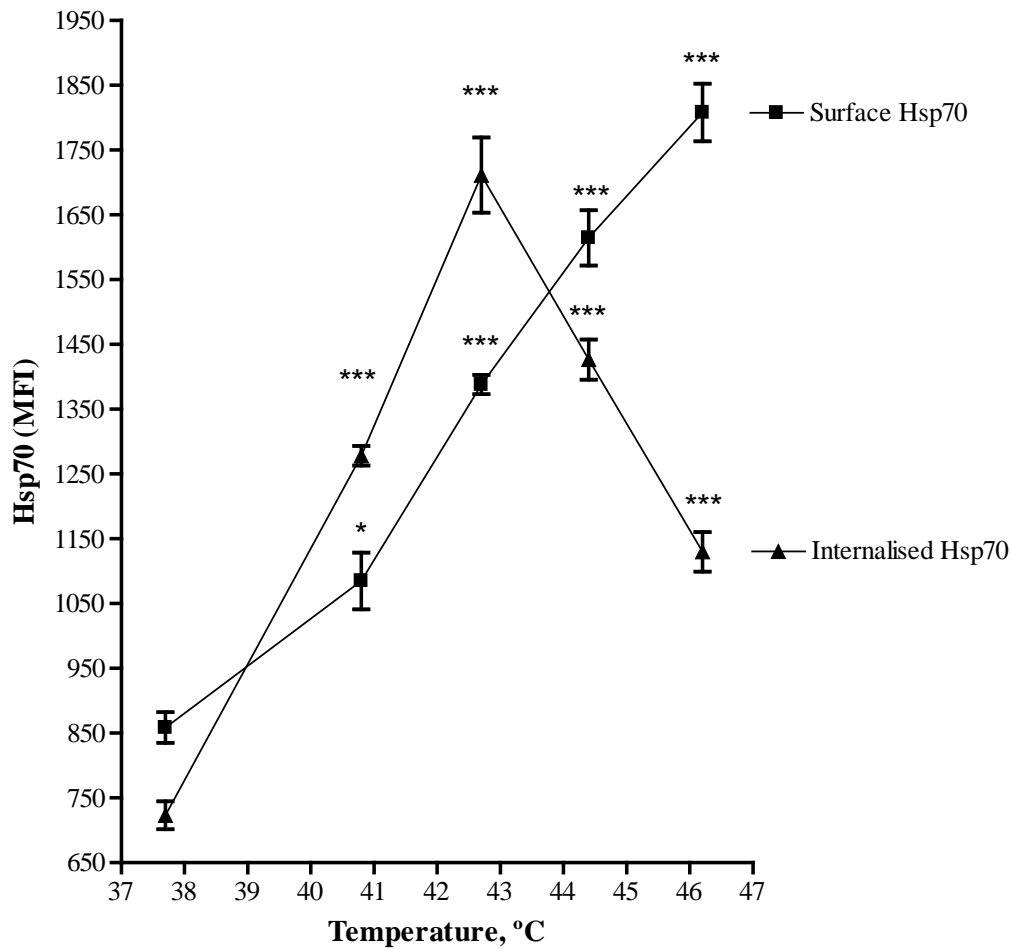
**Figure 4.12: Cell viability (A), caspase-3 (B) and necrosis (C) measurements of U937 cells in response to a 3 hour heat shock treatment at 37.7°C–46.2°C following a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Control: heat shock only (■), 10 µg/mL bovine Hsp70 plus heat shock (▲). Data are presented as SEM, n=3. Significance shown as difference from control between mean temperature points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* (P<0.01), \*\*\* (P<0.001).



**Figure 4.13: Percentage of apoptotic (A) and necrotic (B) U937 cells in response to a 6 hour heat shock treatment at 37.7°C-46.2°C following a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Control: heat shock only (■), 10 µg/mL bovine Hsp70 plus heat shock (▲). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from control between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001).



**Figure 4.14: Surface binding and internalisation of Hsp70 in U937 cells in response to a 3 hour heat shock treatment at 37.7°C–46.2°C following a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from 37.7°C control between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\*\* ( $P<0.001$ ).

#### **4.3.4 Extracellular HSPs inhibit the effects of elevated temperature stress in human leukocytes.**

The protective activity of extracellular bovine Hsp70 was also investigated using human leukocytes induced to apoptosis and necrosis with elevated temperatures as determined in chapter 3. In these experiments, human leukocytes were exposed to heat shock alone at 37.7°C–44.4°C or in combination with 10 µg/mL bovine Hsp70. Apoptosis and necrosis were then quantitatively determined using flow cytometry detecting Annexin V binding (Figure 4.15A, 4.16A, 4.17A), caspase-3 activity (Figure 4.15B, 4.16B, 4.17B) and PI fluorescence (Figure 4.15C, 4.16C, 4.17C).

Apoptosis and necrosis in human leukocytes following treatments with extracellular bovine Hsp70 and elevated temperatures are presented in Figures 4.15-4.17. Surface binding and internalisation of extracellular bovine Hsp70 in human leukocytes following treatments with elevated temperatures are presented in Figure 4.18.

Human leukocytes were incubated at increasing temperatures of 37.7°C–44.4°C for up to two hours, followed by one hour recovery at 37°C. As shown in the temperature response curves in figures 4.15-4.17, when human leukocytes were exposed to temperatures of 40.8°C and 42.7°C a significant increase of apoptotic cell death ( $P<0.001$ ) was observed compared to the 37°C control. Similarly, when exposed to temperatures of 42.7°C and 44.4°C a significant increase of necrotic cell death ( $P<0.05$  to  $P<0.001$ ) was observed compared to the 37°C control (Figures 4.15-4.17). The addition of bovine Hsp70 (10 µg/mL) for two hours at 37°C prior to heat shock treatment resulted in a significant prevention of both apoptosis ( $P<0.05$  to  $P<0.01$  to  $P<0.001$ ) and necrosis ( $P<0.05$  to  $P<0.001$ ) compared to cells exposed to heat shock alone (Figures 4.15-4.17). Extracellular bovine Hsp70 significantly prevented apoptosis (21 % to 8 %) ( $P<0.001$ , Figure 4.15A) of neutrophils at the physiological temperature of 40.8°C compared to cells treated with heat shock alone (Figure 4.15A). At temperatures above 40.8°C there were also significantly less apoptotic neutrophils compared to cells treated with heat shock alone. A pre-treatment with bovine Hsp70 (10 µg/mL) significantly prevented apoptosis of neutrophils at 42.7°C (90 % to 26 %, 215



$P < 0.001$ ), and at  $44.4^{\circ}\text{C}$  (8 % to 4 %,  $P < 0.05$ ) (Figure 4.15A). Furthermore, extracellular bovine Hsp70 also significantly prevented ( $P < 0.01$  to  $P < 0.001$ ) apoptosis of lymphocytes when exposed to elevated temperatures compared to cells exposed to heat shock alone (Figure 4.16A). Apoptosis was significantly prevented at both  $40.8^{\circ}\text{C}$  (6 % to 2 %,  $P < 0.01$ ) and  $42.7^{\circ}\text{C}$  (28 % to 7 %,  $P < 0.001$ ). However, at the increased temperature of  $44.4^{\circ}\text{C}$  there was no significant difference between the percentage of apoptotic lymphocytes pre-incubated with bovine Hsp70 and those exposed to heat shock alone (Figure 4.16A). Similarly, the addition of extracellular bovine Hsp70 ( $10\text{ }\mu\text{g/mL}$ ) to isolated human leukocytes, also significantly prevented apoptosis of monocytes exposed to elevated temperatures compared to cells treated with heat shock alone (Figure 4.17A). Apoptosis of monocytes was prevented at  $40.8^{\circ}\text{C}$  (34 % to 11 %,  $P < 0.001$ ),  $42.7^{\circ}\text{C}$  (51 % to 15 %,  $P < 0.001$ ), and at  $44.4^{\circ}\text{C}$  (30 % to 11 %,  $P < 0.001$ ) (Figure 4.17A). The addition of  $10\text{ }\mu\text{g/mL}$  extracellular bovine Hsp70 for two hours at  $37^{\circ}\text{C}$  prior to heat treatment also significantly prevented caspase-3 activity in neutrophils, 5-fold ( $P < 0.001$ , Figure 4.15B); lymphocytes 4-fold ( $P < 0.001$ , Figure 4.16B); and monocytes 4-fold ( $P < 0.001$ , Figure 4.17B) at all elevated temperatures studied. Finally, as previously determined in chapter 3, as the incubation of human leukocytes with elevated temperatures increased, a decline in apoptotic cell death was observed, concurrently with a significant increase in the percentage of necrotic human leukocytes compared to control cells ( $P < 0.001$ , Figures 4.15-17C). Yet, necrosis of human leukocytes was also found to be prevented as a result of a pre-treatment of cells with extracellular bovine Hsp70 ( $10\text{ }\mu\text{g/mL}$ ) for two hours prior to elevated temperature stress. Necrosis of neutrophils was significantly prevented at  $42.7^{\circ}\text{C}$  (8 % to 3 %,  $P < 0.05$ ), and at  $44.4^{\circ}\text{C}$  (23 % to 9 %,  $P < 0.001$ ) compared to cells exposed to heat shock alone (Figure 4.15C). However, at  $40.8^{\circ}\text{C}$  there was no significant difference between the percentage of necrotic neutrophils pre-incubated with bovine Hsp70 and those exposed to heat shock alone (Figure 4.15C). Necrosis was also prevented in lymphocytes although not until the increased temperature of  $44.4^{\circ}\text{C}$  (35 % to 7 %,  $P < 0.001$ ) compared to cells treated with heat shock alone (Figure 4.16C). At the lower temperatures of  $40.8^{\circ}\text{C}$  and  $42.7^{\circ}\text{C}$  there was no significant difference between the percentage of necrotic lymphocytes pre-incubated with bovine Hsp70 and those exposed to heat shock alone (Figure

4.16C). Furthermore, necrosis was significantly prevented in monocytes ( $P < 0.05$  to  $P < 0.001$ ) compared to cells exposed to heat shock alone (Figure 4.17C). Necrosis in monocytes was prevented at  $42.7^{\circ}\text{C}$  (8 % to 5 %,  $P < 0.05$ ), and at  $44.4^{\circ}\text{C}$  (44 % to 12 %,  $P < 0.001$ ) (Figure 4.17C). Similar to both neutrophils and lymphocytes at  $40.8^{\circ}\text{C}$ , there was no significant difference between the percentage of necrotic monocytes pre-incubated with bovine Hsp70 and those exposed to heat shock alone (Figure 4.17C). These results suggest extracellular bovine Hsp70 (10  $\mu\text{g/mL}$ ) protects neutrophils from apoptosis more effectively than both lymphocytes ( $P < 0.001$ ) and monocytes ( $P < 0.05$  to  $P < 0.001$ ) at temperatures  $> 40.8^{\circ}\text{C}$  (Figures 4.15-4.17A-B), and to protect neutrophils from necrosis more effectively than lymphocytes ( $P < 0.05$  to  $P < 0.001$ ) and monocytes ( $P < 0.001$ ) at temperatures  $> 42.7^{\circ}\text{C}$  (Figures 4.15-4.17C).

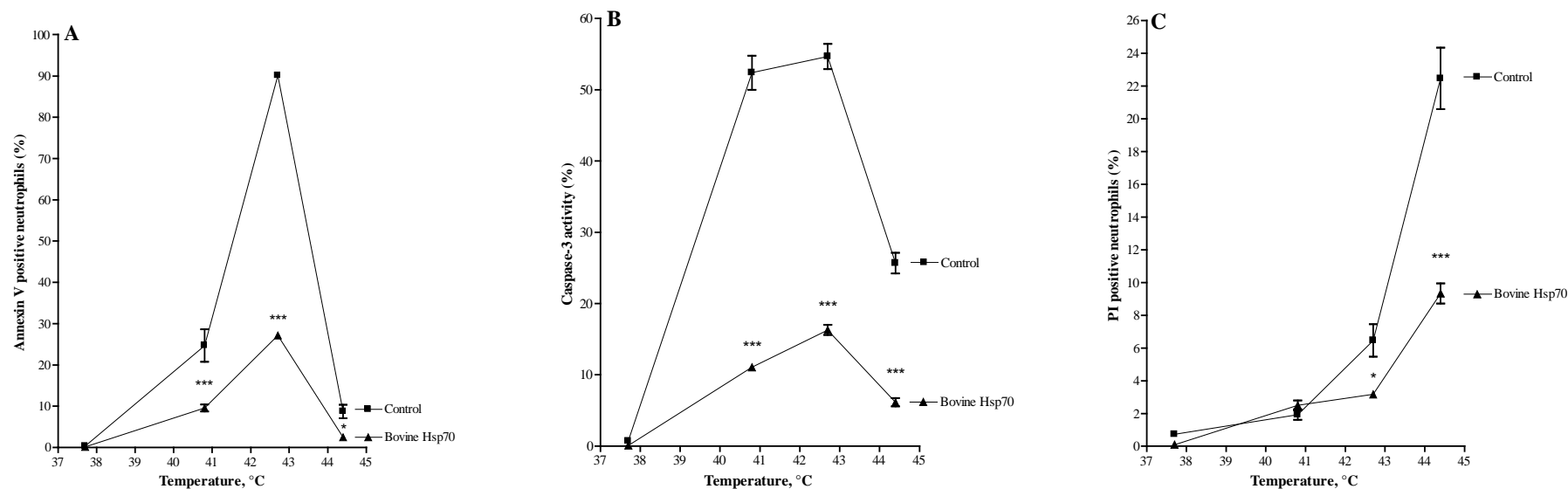
#### **4.3.4.1 Binding and internalisation of extracellular HSPs following elevated temperature stress in human leukocytes.**

The binding and internalisation of extracellular bovine Hsp70 (10  $\mu\text{g/mL}$ ) in human leukocytes pre-incubated for two hours at  $37^{\circ}\text{C}$  and following exposure to elevated temperatures ( $37.7^{\circ}\text{C}$ – $44.4^{\circ}\text{C}$ ) for up to two hours was investigated. A significant increase in surface binding ( $P < 0.05$  to  $P < 0.01$  to  $P < 0.001$ , Figure 4.18A) and internalisation ( $P < 0.05$  to  $P < 0.01$  to  $P < 0.001$ , Figure 4.18B) of bovine Hsp70 was observed compared to control in all leukocyte subtypes, at all temperatures studied, with the exception of surface bound Hsp70 on neutrophils at  $40.8^{\circ}\text{C}$ , where a non-significant increase was observed (Figure 4.18A). A significant increase of binding was seen on neutrophils, 1-fold ( $P < 0.01$ ) at  $42.7^{\circ}\text{C}$ , and an almost 2-fold increase ( $P < 0.01$ ) at  $44.4^{\circ}\text{C}$  compared to control cells (Figure 4.18A). Binding also increased on lymphocytes, 1-fold ( $P < 0.01$ ) at  $40.8^{\circ}\text{C}$ , 2-fold ( $P < 0.001$ ) at  $42.7^{\circ}\text{C}$  and 2-fold ( $P < 0.001$ ) at  $44.4^{\circ}\text{C}$  compared to control cells (Figure 4.18A). Furthermore, binding on monocytes increased compared to control, 1-fold ( $P < 0.05$ ) at  $40.8^{\circ}\text{C}$ , almost 2-fold ( $P < 0.001$ ) at  $42.7^{\circ}\text{C}$  and 3-fold ( $P < 0.001$ ) at  $44.4^{\circ}\text{C}$ .

Internalised bovine Hsp70 in neutrophils also increased 1-fold ( $P < 0.01$ ) at  $40.8^{\circ}\text{C}$ , 2-fold ( $P < 0.001$ ) at  $42.7^{\circ}\text{C}$  but decreased 1-fold ( $P < 0.01$ ) at  $44.4^{\circ}\text{C}$ , however levels did not reduce to equal those of the control (Figure 4.18B). Internalised bovine Hsp70 also increased in lymphocytes, almost 2-fold

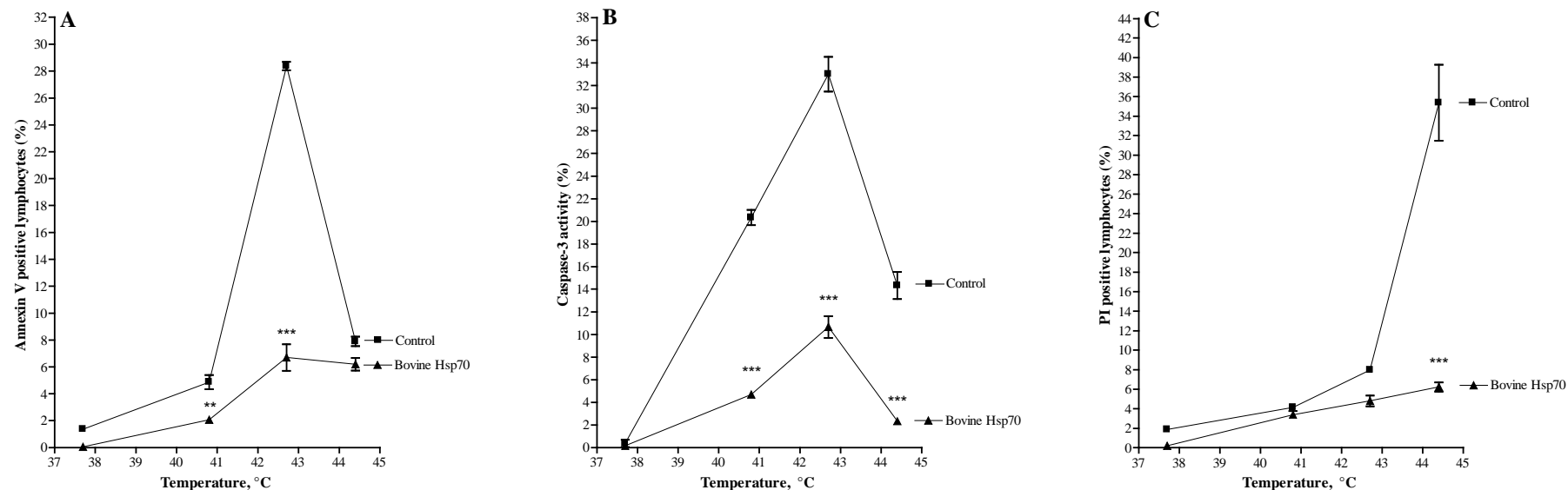
( $P < 0.001$ ) at 40.8°C, 2-fold ( $P < 0.001$ ) at 42.7°C but decreased almost 2-fold at 44.4°C similar to neutrophils, and levels did not reduce to equal those of the control, however these results were found to be non-significant (Figure 4.18B). Internalised bovine Hsp70 also increased in monocytes, 1-fold ( $P < 0.01$ ) at 40.8°C, 1-fold ( $P < 0.001$ ) at 42.7°C but decreased 1-fold ( $P < 0.01$ ) at 44.4°C similar to both neutrophils and lymphocytes, however levels did not reduce to equal those of the control (Figure 4.18B). At temperatures of 40.8°C and above, a significantly ( $P < 0.001$ ) greater amount of bovine Hsp70 was detected on the cell surface of monocytes and lymphocytes compared to internalisation (Figure 4.18A-B), and at temperatures of 42.7°C and above, a significantly ( $P < 0.01$  to  $P < 0.001$ ) greater amount of bovine Hsp70 was detected on the cell surface of neutrophils compared to internalisation (Figure 4.18).

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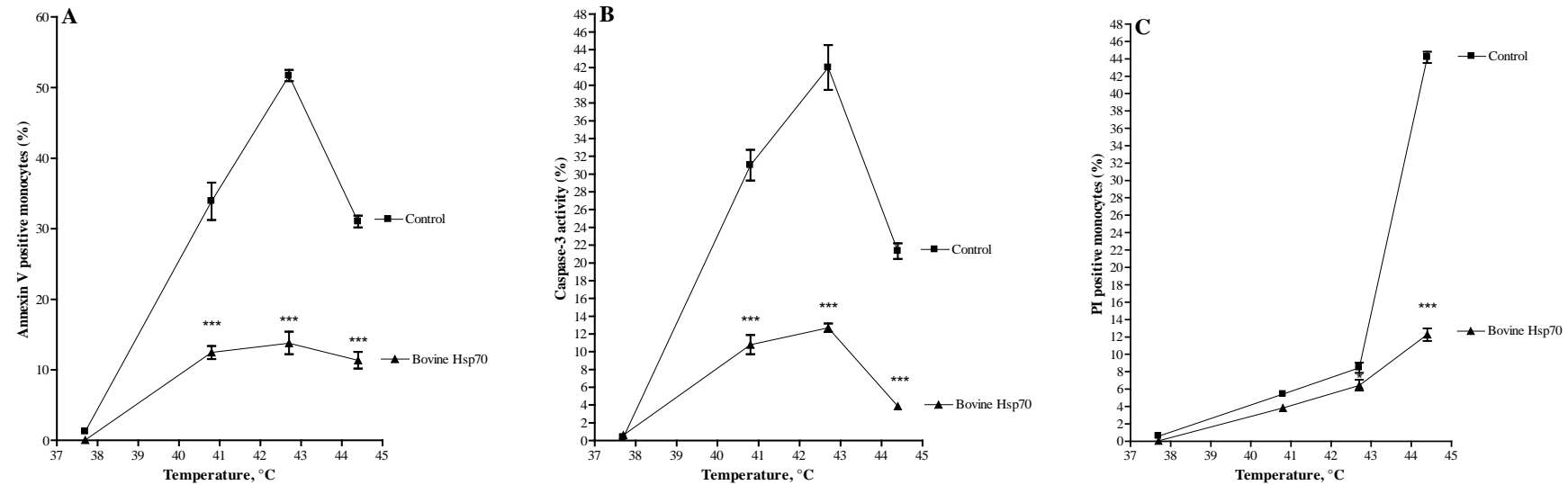
**Figure 4.15: Percentage of Annexin V (A), caspase-3 (B) and PI (C) positive neutrophils in response to a 2 hour heat shock treatment at 37.7°C–44.4°C following a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Control: heat shock only (■), 10 µg/mL bovine Hsp70 plus heat shock (▲). Data are presented as mean ± SEM, n=3. Significance shown as difference from control between mean temperature points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\*\* (P<0.001).



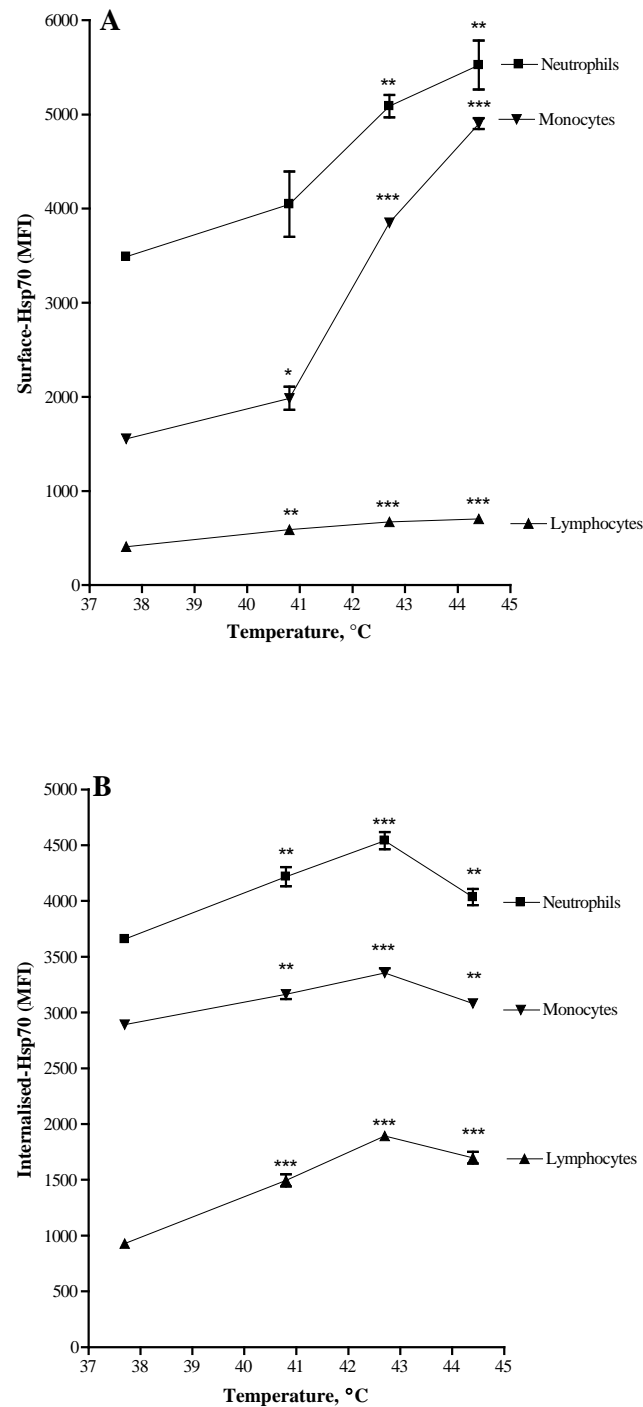
**Figure 4.16: Percentage of Annexin V (A), caspase-3 (B) and PI (C) positive lymphocytes in response to a 2 hour heat shock treatment at 37.7°C–44.4°C following a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Control: heat shock only (■), 10 µg/mL bovine Hsp70 plus heat shock (▲). Data are presented as mean ± SEM, n=3. Significance shown as difference from control between mean temperature points though use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* (P<0.01), \*\*\* (P<0.001).



**Figure 4.17: Percentage of Annexin V (A), caspase-3 (B) and PI (C) positive monocytes in response to a 2 hour heat shock treatment at 37.7°C–44.4°C following 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Control: heat shock only (■), 10 µg/mL bovine Hsp70 plus heat shock (▲). Data are presented as mean ± SEM, n=3. Significance shown as difference from control between mean temperature points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\*\* (P<0.001).



**Figure 4.18: Surface binding (A) and internalisation (B) of Hsp70 in human leukocytes in response to a 2 hour heat shock treatment at 37.7°C–44.4°C following a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=4$ . Significance shown as difference from 37.7°C control between mean temperature points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).

#### **4.3.5 Potential receptors for extracellular bovine Hsp70 on U937 cells.**

The cyto-protective functions of extracellular bovine Hsp70 as seen in the previous experiments are probably activated through its interaction with a receptor on the cell surface. Previous studies have indeed suggested Hsp70 to interact with cultured cells and primary cells through several surface receptors including that of SRs. Thus, the final part of the study was designed to investigate SRs as potential Hsp70 receptors on the surface of U937 cells and human leukocytes.

Detection of Hsp70 surface receptors on U937 cells are presented in Figures 4.19-4.24 and Tables 4.5-4.6.

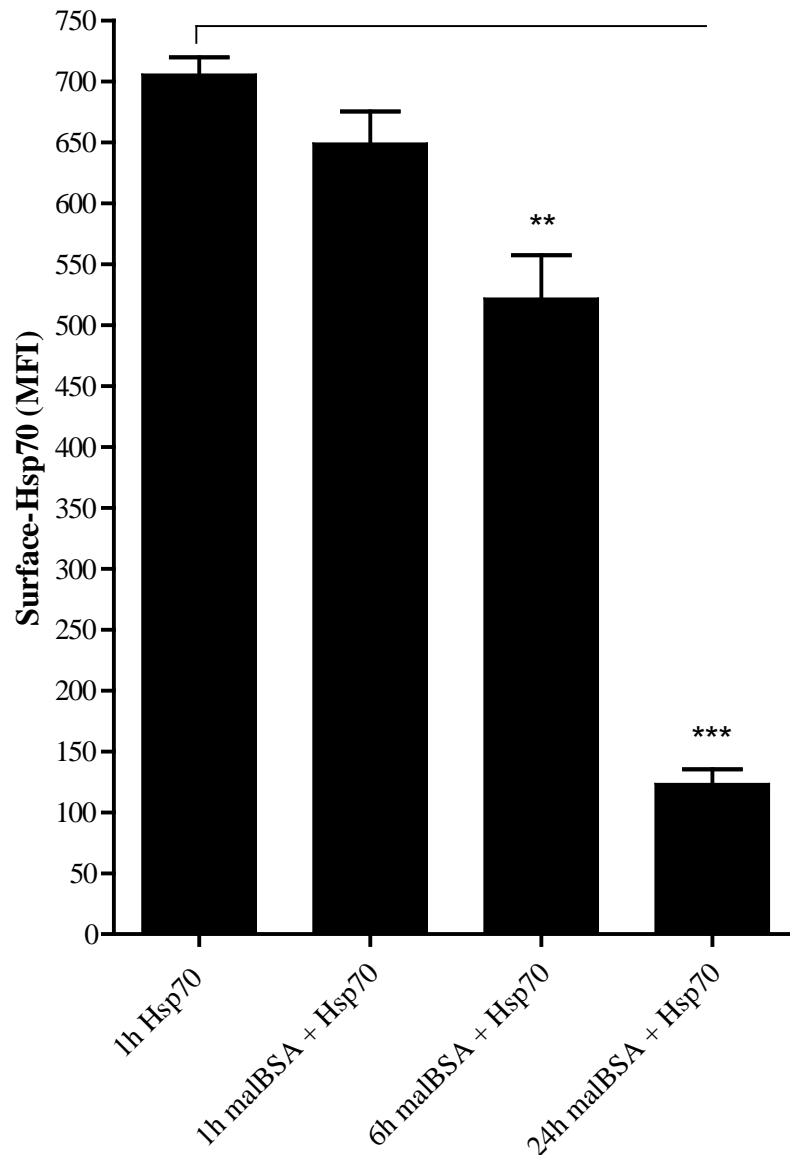
Potential membrane receptors on U937 cells were identified through competition experiments, exposing cells to bovine Hsp70 (10 µg/mL) for one hour at 37°C alone or in combination with a pre-incubation of malBSA, fucoidan or poly (I) for up to twenty-four hours or Annexin V for twenty minutes at 37°C. U937 cells incubated with bovine Hsp70 (10 µg/mL) for one hour at 37°C was readily detected on the cell surface by flow cytometry (Figures 4.19-4.22). In comparison, U937 cells exposed to malBSA, fucoidan or poly (I) for up to twenty four hours prior to incubation with bovine Hsp70 significantly inhibited the binding of Hsp70 to U937 cells ( $P<0.01$  to  $P<0.001$ , Figures 4.19-4.21). However, pre-incubation of cells for twenty minutes with Annexin V did not prevent binding of bovine Hsp70 to the U937 cell surface (Figure 4.22B). MalBSA, a chemically modified BSA protein which reports suggest binds with high affinity to several SRs, significantly suppressed the binding of bovine Hsp70 after a six ( $P<0.01$ ), and twenty-four ( $P<0.001$ ) hour pre-incubation compared to control cells (Figure 4.19). However, following a one hour incubation of U937 cells with malBSA prior to treatment with bovine Hsp70 did not significantly reduce Hsp70 binding to the U937 cell surface (Figure 4.19). These results provided initial evidence for the existence of SRs on U937 cells, however to identify which SRs may be involved two SR ligands, fucoidan and poly (I) were utilised. Fucoidan, in a similar action to malBSA significantly reduced the binding of bovine Hsp70 after a six ( $P<0.01$ ), and twenty-four ( $P<0.001$ ) hour pre-incubation compared to control cells (Figure 4.20).



Furthermore fucoidan also significantly reduced the binding of bovine Hsp70 to U937 cells after a one hour pre-incubation compared to control cells (Figure 4.20). Similarly, poly (I) significantly inhibited the binding of bovine Hsp70 after a one ( $P<0.001$ ), six ( $P<0.001$ ), and twenty-four ( $P<0.001$ ) hour pre-incubation compared to control cells (Figure 4.21). PS has previously been demonstrated as a potential binding site for Hsp72 and Hsc70 (Arispe et al. 2004), however PS should only be present on the surface of senescent U937 cells, thus to rule out PS as a potential binding site for extracellular bovine Hsp70 at 37°C, Annexin V was utilised. PS was not detected on the surface of U937 cells at 37°C using Annexin V by flow cytometry (Figure 4.22A). In addition, pre-incubation of U937 cells with bovine Hsp70 did not significantly affect PS detection on U937 cells (Figure 4.22A). Furthermore, pre-incubation of U937 cells with Annexin V did not significantly inhibit Hsp70 binding to U937 cells (Figure 4.22B). In contrast, on U937 cells exposed to a one hour heat shock treatment at 42°C, PS was easily detectable compared to control cells ( $P<0.001$ , Figure 4.22A). This detection was significantly reduced by a pre-incubation with bovine Hsp70 prior to incubation with Annexin V ( $P<0.001$ , Figure 4.22A), but was unaffected by the addition of bovine Hsp70 following the pre-incubation with Annexin V (Figure 4.22A). On these heat shocked cells, a pre-incubation with Annexin V prior to incubation with bovine Hsp70 significantly inhibited the binding of bovine Hsp70 to U937 cells compared to control cells ( $P<0.001$ , Figure 4.22B), however was unaffected by the addition of Annexin V following the pre-incubation with bovine Hsp70 (Figure 4.22B), indicating PS as a potential binding site for extracellular bovine Hsp70 under conditions of elevated temperature stress.

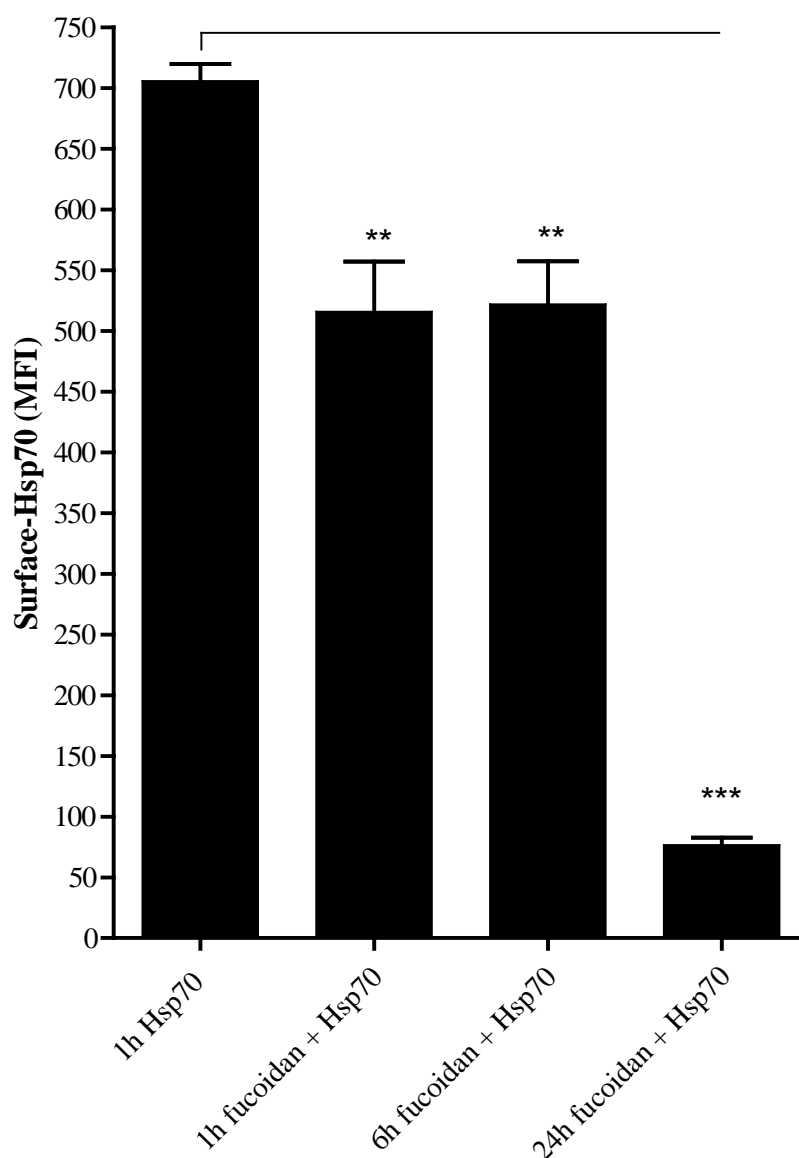
To identify which SRs might be involved in the binding of extracellular bovine Hsp70 to U937 cells at 37°C and following a one hour heat shock treatment at 42°C, antibodies against the SRs: LOX-1 and SR-A1, along with secondary R-PE or FITC conjugated antibodies were utilised for indirect detection. As shown in the histograms of figure 4.23 and as revealed in table 4.5, LOX-1 was not detected on the surface of U937 cells at 37°C compared to no stain control cells using anti-LOX-1 by flow cytometry (Figure 4.23; Table 4.5). Furthermore, on U937 cells exposed to a one hour heat shock treatment at 42°C, LOX-1 was

again not detectable compared to no stain control cells (Figure 4.23; Table 4.5). In contrast, SR-A1 was easily detected on the surface of U937 cells at 37°C, as indicated by a significant increase of MFI compared to no stain control cells using anti-SR-A1 by flow cytometry ( $P < 0.001$ , Figure 4.24, Table 4.6). On U937 cells exposed to a one hour heat shock treatment at 42°C, SR-A1 expression was significantly up-regulated compared to no stain control cells ( $P < 0.001$ ), and to the cells incubated at 37°C ( $P < 0.001$ , Figure 4.24, Table 4.6), which confirmed the presence of SR-A1 receptors on U937 cell surface (Figure 4.24, Table 4.6).



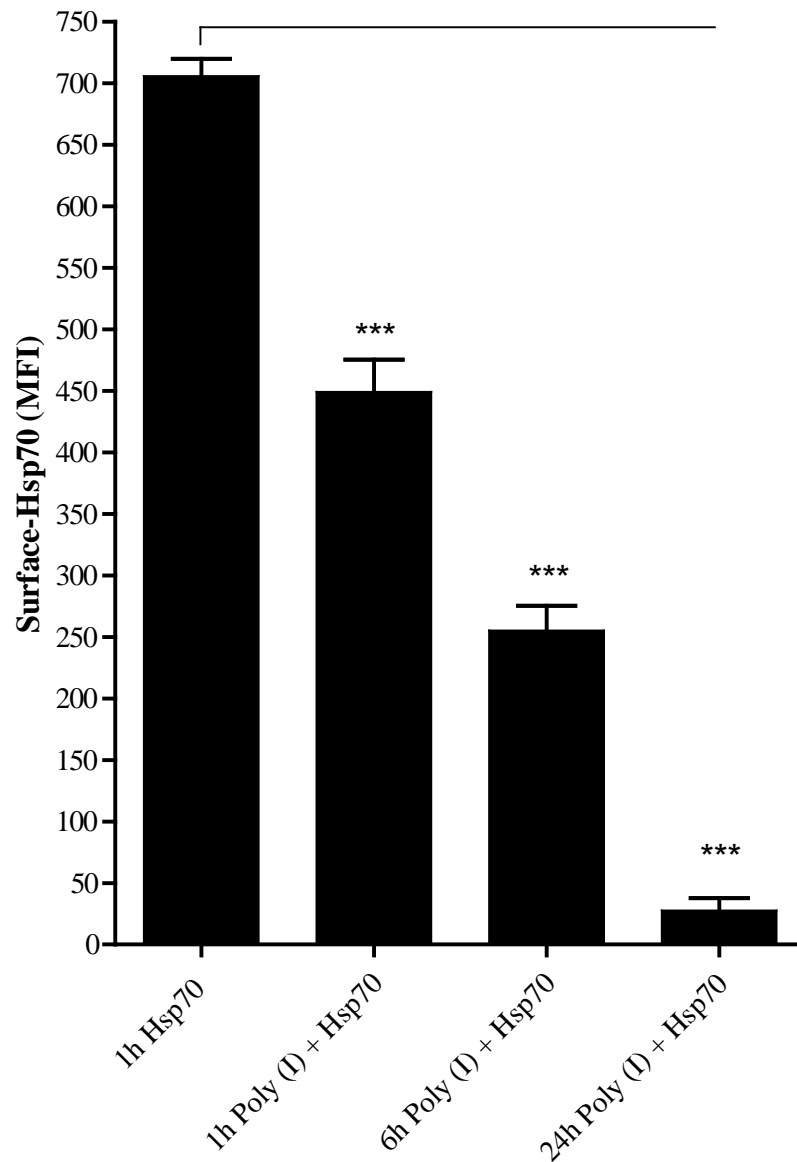
**Figure 4.19: The effect of malBSA on the binding of bovine Hsp70 to the surface of U937 cells.**

U937 cells were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C, following pre-incubation with 10  $\mu\text{g/mL}$  malBSA for 1, 6 or 24 hours at 37°C. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from Hsp70 control between mean time points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



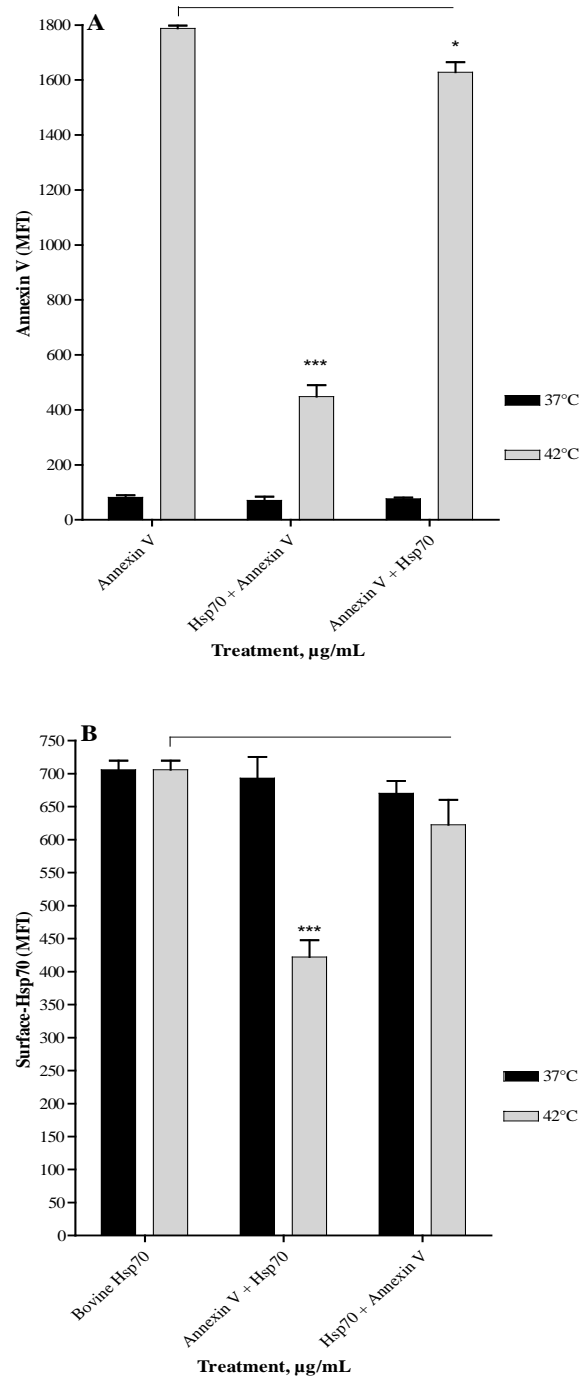
**Figure 4.20: The effect of fucoidan on the binding of bovine Hsp70 to the surface of U937 cells.**

U937 cells were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C, following pre-incubation with 10  $\mu\text{g/mL}$  fucoidan for 1, 6 or 24 hours at 37°C. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from Hsp70 control between mean time points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



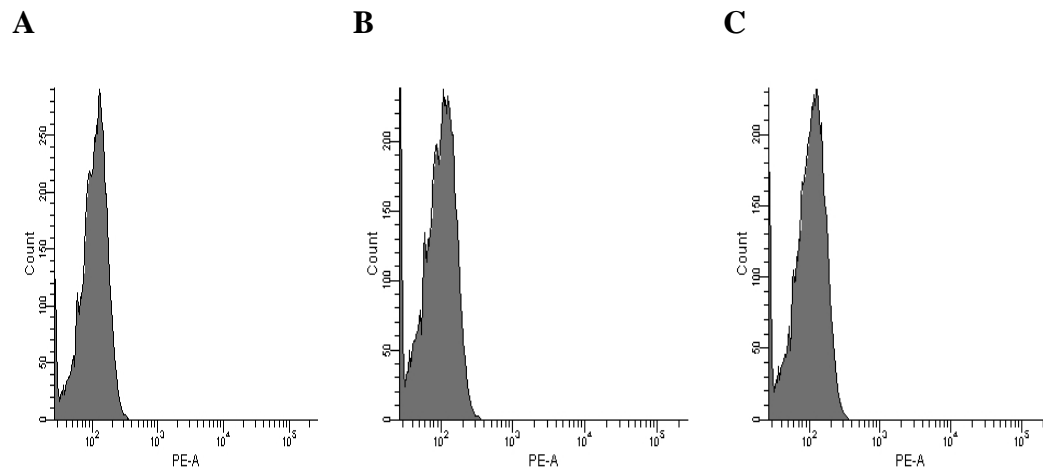
**Figure 4.21: The effect of poly (I) on the binding of bovine Hsp70 to the surface of U937 cells.**

U937 cells were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 at 37°C for 1 hour, following pre-incubation with 10  $\mu\text{g/mL}$  poly (I) for 1, 6 or 24 hours at 37°C. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from Hsp70 control between mean time points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P < 0.001$ ).



**Figure 4.22: Binding of bovine Hsp70 to PS on the surface of U937 cells at 37°C and in response to a 1 hour 42°C heat shock treatment.**

Either: U937 cells were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C, following pre-incubation with Annexin V for 20 minutes at 37°C. Or U937 cells were incubated with Annexin V for 20 minutes at 37°C, following pre-incubation with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C. **(A)** Annexin V surface binding. **(B)** Hsp70 surface binding. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from Annexin V or Hsp70 control between mean treatments through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\*\* ( $P<0.001$ ).



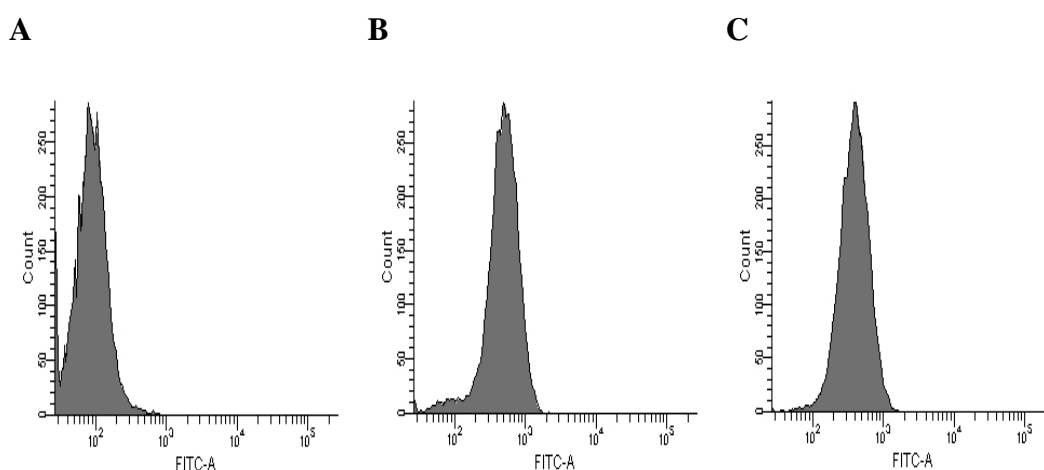
**Figure 4.23: LOX-1 analysis of U937 cells using flow cytometry.**

U937 cells were gated using FSC/SSC. (A) No stain control sample, (B) LOX-1 expression at 37°C and (C) LOX-1 expression at 42°C. Data are representative of three independent experiments and 10,000 events were recorded.

**Table 4.5: Expression of LOX-1 on U937 cells at 37°C and in response to 1 hour 42°C heat shock treatment.**

Percentage of the total population of U937 cells positive for LOX-1 mean ( $\pm$ SEM, n=3)		
LOX-1 Expression	Control	11 % $\pm$ 0.9
	No Treatment	12 % $\pm$ 1.0
	Heat Shock Treatment	17 % $\pm$ 1.0

Note: Control = no stain control sample. No treatment = 37°C. Heat shock treatment = 1 hour at 42°C. Seeding density  $3 \times 10^5$ . Significance shown as difference from no stain control between mean treatments through use of one- way ANOVA with Bonferroni's multiple comparison *post hoc* test.



**Figure 4.24: SR-A1 analysis of U937 cells using flow cytometry.**

U937 cells were gated using FSC/SSC. (A) No stain control sample, (B) SR-A1 expression at 37°C and (C) SR-A1 expression at 42°C. Data are representative of three independent experiments and 10,000 events were recorded.

**Table 4.6: Expression of SR-A1 on U937 cells at 37°C and in response to 1 hour 42°C heat shock treatment.**

Percentage of the total population of U937 cells positive for SR-A1 mean (± SEM, n=3)		
SR-A1 Expression	Control	14 % ± 1.2
	No Treatment	58 % ± 3.9 ***
	Heat Shock Treatment	71 % ± 2.3 ***

Note: Control = no stain control sample. No treatment = 37°C. Heat shock treatment = 1 hour at 42°C. Seeding density  $3 \times 10^5$ . Significance shown as difference from no stain control between mean treatments through use of one- way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).



#### **4.3.6 Potential receptors for extracellular bovine Hsp70 on human leukocytes.**

Detection of Hsp70 surface receptors on neutrophils, lymphocytes and monocytes are presented in Figures 4.25-4.34.

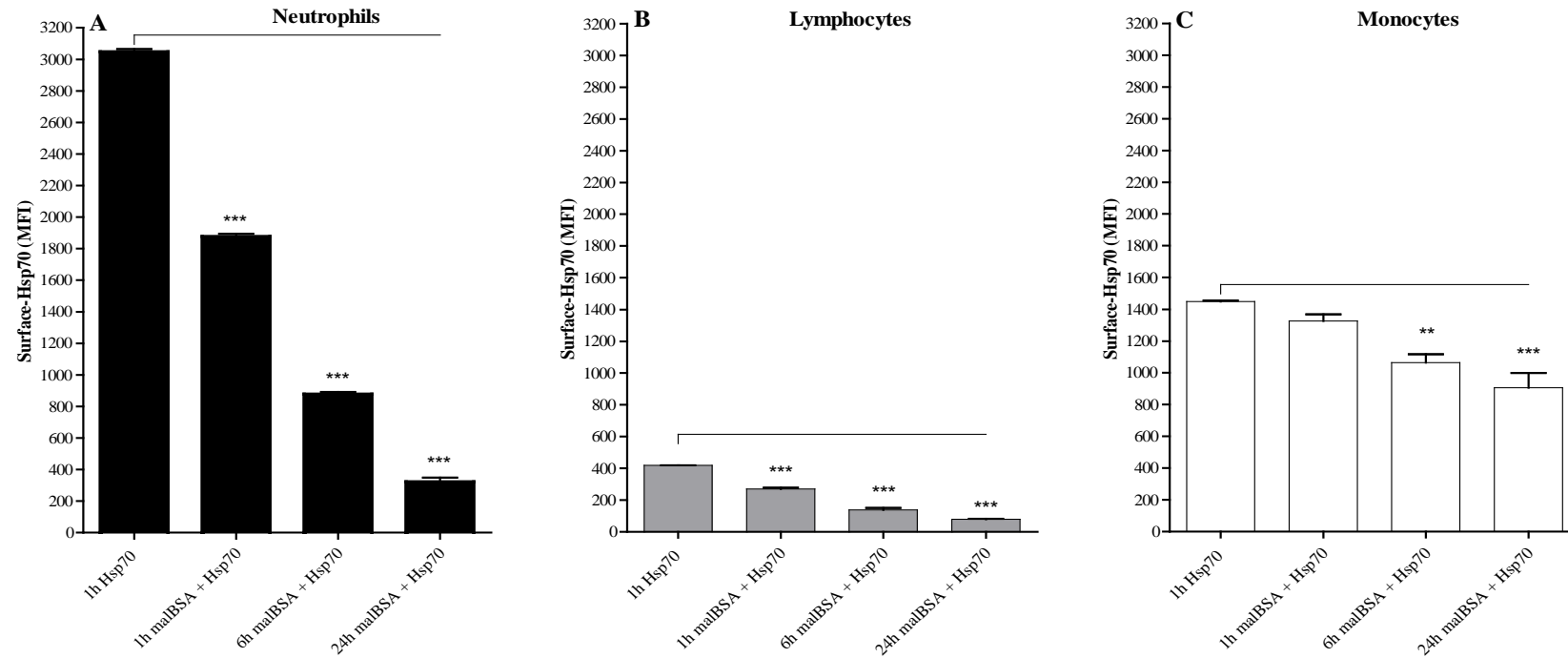
Potential membrane receptors on human leukocytes, similar to U937 cells were identified through competition experiments, exposing cells to 10 µg/mL bovine Hsp70 for one hour at 37°C alone or in combination with a pre-incubation of malBSA, fucoidan or poly (I) for up to twenty-four hours or Annexin V for twenty minutes at 37°C. Hsp70 was readily detected on the surface of human leukocytes following incubation with bovine Hsp70 for one hour at 37°C (Figures 4.25-4.27). In contrast, human leukocytes exposed to malBSA, fucoidan or poly (I) for up to twenty-four hours prior to incubation with bovine Hsp70 significantly inhibited the binding of Hsp70 to human leukocytes ( $P < 0.01$  to  $P < 0.001$ , Figures 4.25-4.27). However, pre-incubation of cells for twenty minutes with Annexin V did not prevent binding of bovine Hsp70 to the human leukocytes cell surface (Figure 4.28-4.30). MalBSA significantly suppressed the binding of bovine Hsp70 to both neutrophils ( $P < 0.001$ , Figure 4.25A) and lymphocytes ( $P < 0.001$ , Figure 4.25B) after a one ( $P < 0.001$ ), six ( $P < 0.001$ ), and twenty-four ( $P < 0.001$ ) hour pre-incubation compared to control cells (Figures 4.25A-B). Furthermore, malBSA also significantly suppressed the binding of bovine Hsp70 to monocytes but after a six ( $P < 0.01$ ), and twenty-four ( $P < 0.001$ ) hour pre-incubation compared to control cells (Figure 4.25C). Similar to U937 cells, a one hour incubation of monocytes with malBSA prior to treatment with bovine Hsp70 did not significantly reduce Hsp70 binding to monocytes (Figure 4.25C). Since malBSA binds with high affinity to SRs, these results provided evidence for the existence of SRs on human leukocytes. In order to identify which SRs may be involved two SR ligands, fucoidan and poly (I) were utilised. Fucoidan, significantly reduced the binding of bovine Hsp70 on neutrophils ( $P < 0.001$ , Figure 4.26A), lymphocytes ( $P < 0.001$ , Figure 4.26B) and monocytes ( $P < 0.001$ , Figure 4.26C), after a six ( $P < 0.001$ ) and twenty-four ( $P < 0.001$ ) hour pre-incubation compared to control cells (Figure 4.26A-C). Furthermore, fucoidan also significantly suppressed the binding of bovine Hsp70 to lymphocytes ( $P < 0.001$ ) after a one hour pre-incubation compared to control cells

(Figure 4.26B). However, on both neutrophils and monocytes after a one hour incubation with fucoidan prior to treatment with bovine Hsp70 there was no significant reduction of Hsp70 binding compared to control cells (Figure 4.26A, C). In a similar action to that of malBSA, poly (I) also significantly blocked the binding of extracellular bovine Hsp70 to both neutrophils ( $P<0.001$ , Figure 4.27A) and lymphocytes ( $P<0.001$ , Figure 4.27B) after a one ( $P<0.001$ ), six ( $P<0.001$ ) and twenty-four ( $P<0.001$ ) hour pre-incubation compared to control cells (Figures 4.27A-B). In addition, poly (I) also significantly suppressed the binding of bovine Hsp70 to monocytes but after a six ( $P<0.001$ ) and twenty-four ( $P<0.001$ ) hour pre-incubation compared to control cells (Figure 4.27C). However, a one hour incubation of monocytes with poly (I) prior to treatment with bovine Hsp70 did not significantly reduce Hsp70 binding to monocytes (Figure 4.27C).

In a similar method to that used with U937 cells, PS was also examined as a potential binding site for extracellular bovine Hsp70 at 37°C or 42°C using Annexin V. PS was not detected on the surface of neutrophils (Figure 4.28A), lymphocytes (Figure 4.29A) or monocytes (Figure 4.30A) at 37°C using Annexin V by flow cytometry, in addition pre-incubation of human leukocytes with bovine Hsp70 did not significantly affect PS detection on human leukocytes (Figures 4.28-4.30A). Furthermore, pre-incubation of human leukocytes with Annexin V did not significantly inhibit Hsp70 binding to human leukocytes (Figures 4.28-4.30B). In contrast, on human leukocytes exposed to a one hour heat shock treatment at 42°C, PS was easily detectable compared to control cells ( $P<0.001$ , Figures 4.28-4.30A), which was significantly reduced by a pre-incubation with bovine Hsp70 prior to incubation with Annexin V on neutrophils ( $P<0.001$ , Figure 4.28A), lymphocytes ( $P<0.001$ , Figure 4.29A) and monocytes ( $P<0.001$ , Figure 4.30A), but was unaffected by the addition of bovine Hsp70 following the pre-incubation with Annexin V (Figures 4.28-4.30A). Furthermore, on these cells a pre-incubation with Annexin V prior to incubation with bovine Hsp70 significantly inhibited the binding of bovine Hsp70 to neutrophils ( $P<0.001$ , Figure 4.28B), lymphocytes ( $P<0.001$ , Figure 4.29B) and monocytes (Figure 4.30B) compared to control cells, however was unaffected by the addition of Annexin V following pre-incubation with bovine Hsp70 (Figures

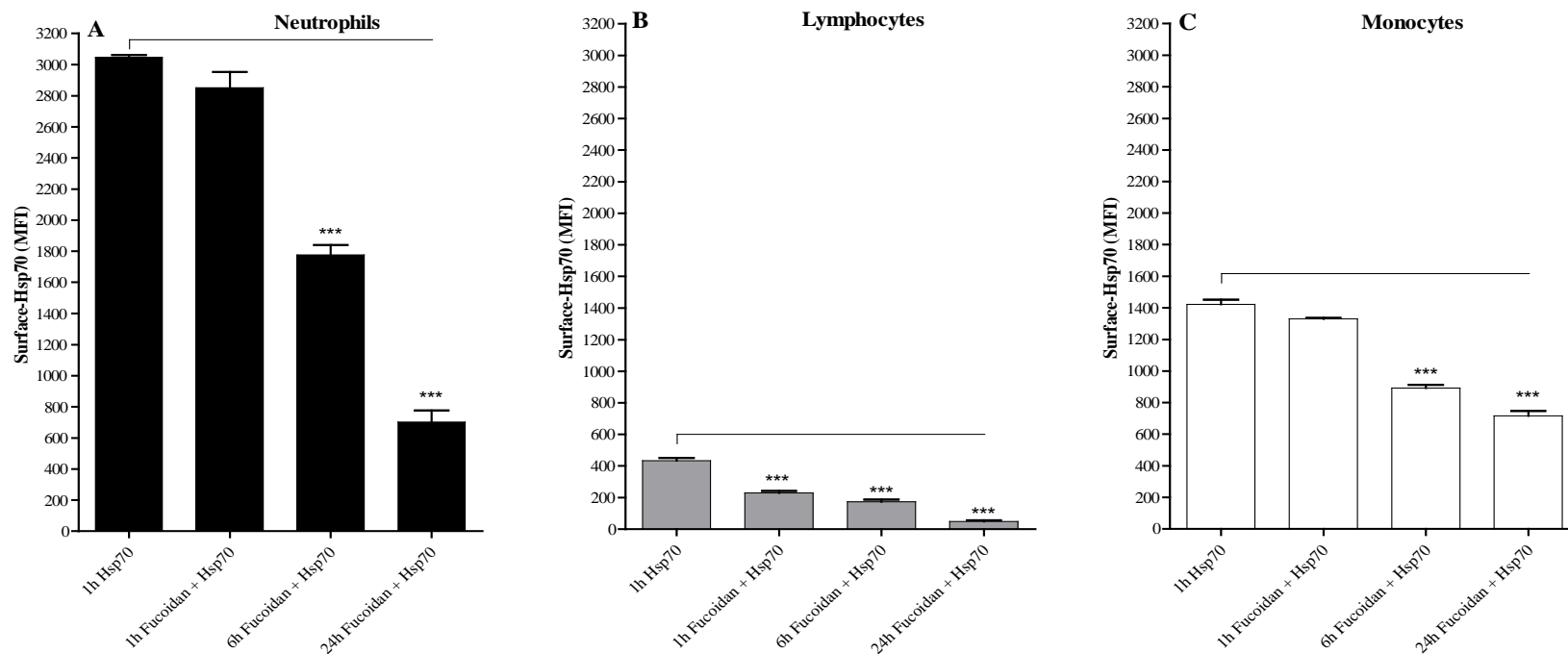
4.28-4.30B), indicating PS as a potential binding site for extracellular bovine Hsp70 under conditions of elevated temperature stress.

To identify which SRs might be involved in the binding of extracellular bovine Hsp70 to human leukocytes at 37°C and following a one hour heat shock treatment at 42°C, antibodies against the SRs: LOX-1 and SR-A1, along with secondary R-PE or FITC conjugated antibodies were utilised for indirect detection. As shown in the histograms of figure 4.31 and the bar chart of figure 4.32, LOX-1 was easily detected on the surface of neutrophils (41 % positive for LOX-1), and to a lesser degree on both lymphocytes (6.5 % positive for LOX-1) and monocytes (5.3 % positive for LOX-1) at 37°C compared to no stain control cells using anti-LOX-1 by flow cytometry (Figures 4.31-4.32). Furthermore, neutrophils expressed a significantly greater amount of LOX-1 on their cell surface compared to both lymphocytes ( $P<0.001$ ) and monocytes ( $P<0.001$ , Figures 4.31-4.32) at 37°C. On human leukocytes exposed to a one hour heat shock treatment at 42°C, LOX-1 was significantly up-regulated, 28-fold on neutrophils ( $P<0.001$ ), 40-fold on lymphocytes ( $P<0.001$ ) and surprisingly the most significant up-regulation was on monocytes, a 77-fold increase ( $P<0.001$ ) compared to no stain control cells and to the cells incubated at 37°C (Figures 4.31-4.32). Similarly, as revealed in figures 4.33-4.34 SR-A1 was also easily detected on the surface of neutrophils (29 % positive for SR-A1) and monocytes (43 % positive for SR-A1) and to a lesser degree on lymphocytes (6 % positive for SR-A1) at 37°C, as indicated by a significant increase in MFI compared to no stain control cells using anti-SR-A1 by flow cytometry (Figures 4.33-4.34). Furthermore, monocytes expressed a significantly greater amount of SR-A1 on their cell surface compared to both neutrophils ( $P<0.001$ ) and lymphocytes ( $P<0.001$ , Figures 4.33-4.34) at 37°C. On human leukocytes exposed to a one hour heat shock treatment at 42°C, SR-A1 expression was significantly up-regulated, 68-fold on neutrophils ( $P<0.001$ ), 7-fold on lymphocytes ( $P<0.001$ ) and 49-fold on monocytes ( $P<0.001$ ) compared to no stain control cells and to the cells incubated at 37°C (Figures 4.33-4.34). These data confirm the presence of LOX-1 and SR-A1 receptors on human leukocytes cell surfaces (Figure 4.31-4.34).



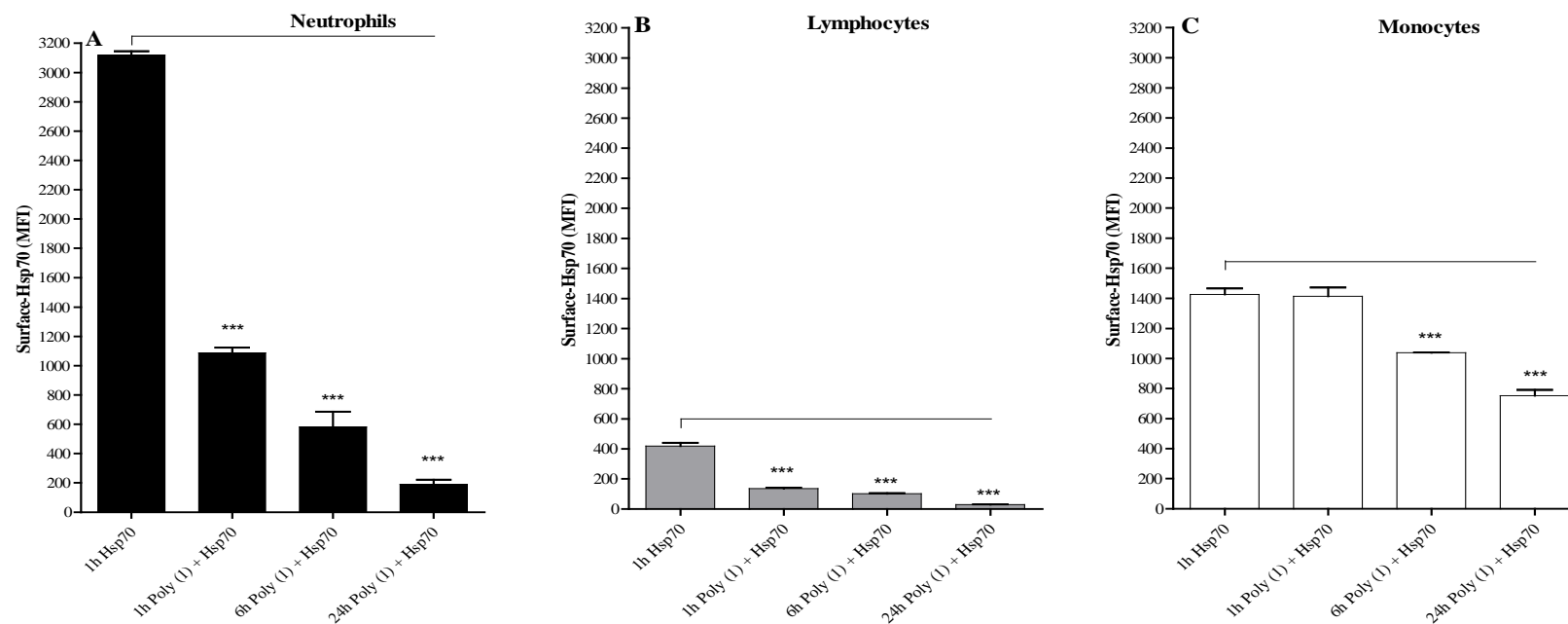
**Figure 4.25: The effect of malBSA on the binding of bovine Hsp70 to the surface of human leukocytes.**

Human leukocytes were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 at 37°C for 1 hour, following pre-incubation with malBSA for 1, 6 or 24 hours at 37°C. Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from Hsp70 control between mean time points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



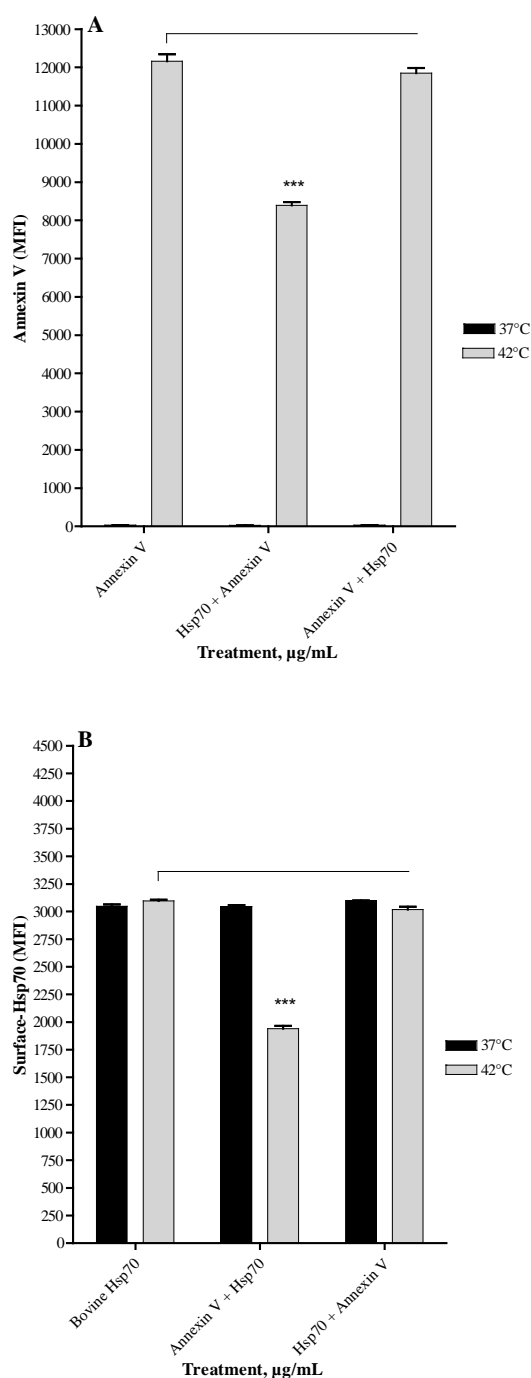
**Figure 4.26: The effect of fucoidan on the binding of bovine Hsp70 to the surface of human leukocytes.**

Human leukocytes were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 at 37°C for 1 hour, following pre-incubation with fucoidan for 1, 6 or 24 hours at 37°C. Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from Hsp70 control between mean time points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



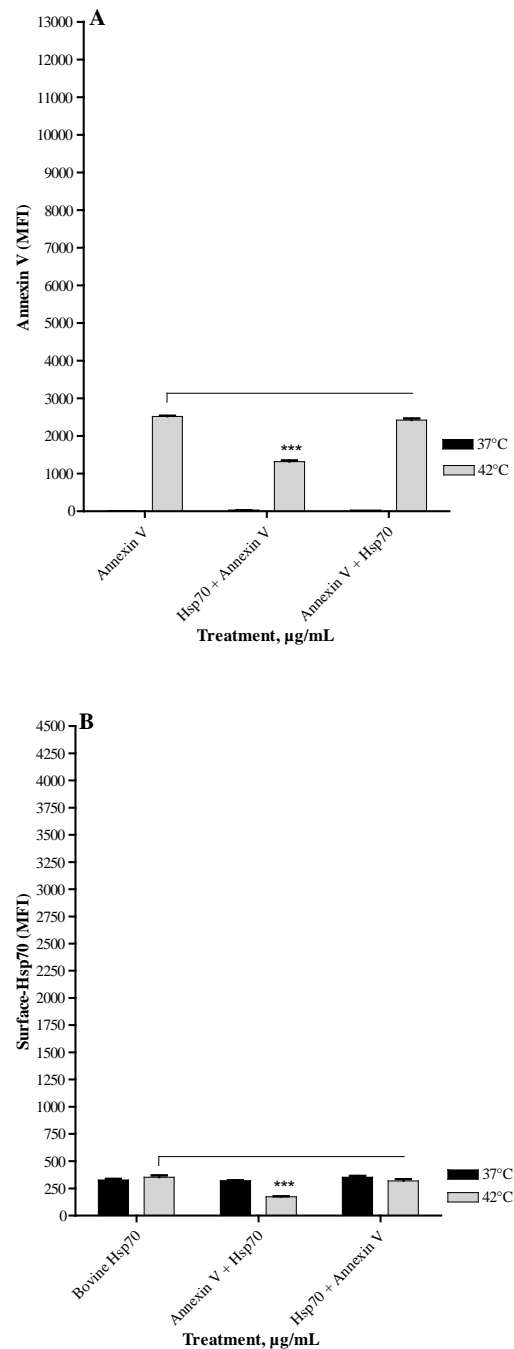
**Figure 4.27: The effect of poly (I) on the binding of bovine Hsp70 to the surface of human leukocytes.**

Human leukocytes were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 at 37°C for 1 hour, following pre-incubation with poly (I) for 1, 6 or 24 hours at 37°C. Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from Hsp70 control between mean time points through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).



**Figure 4.28: Binding of bovine Hsp70 to PS on the surface of neutrophils at 37°C and in response to a 1 hour 42°C heat shock treatment.**

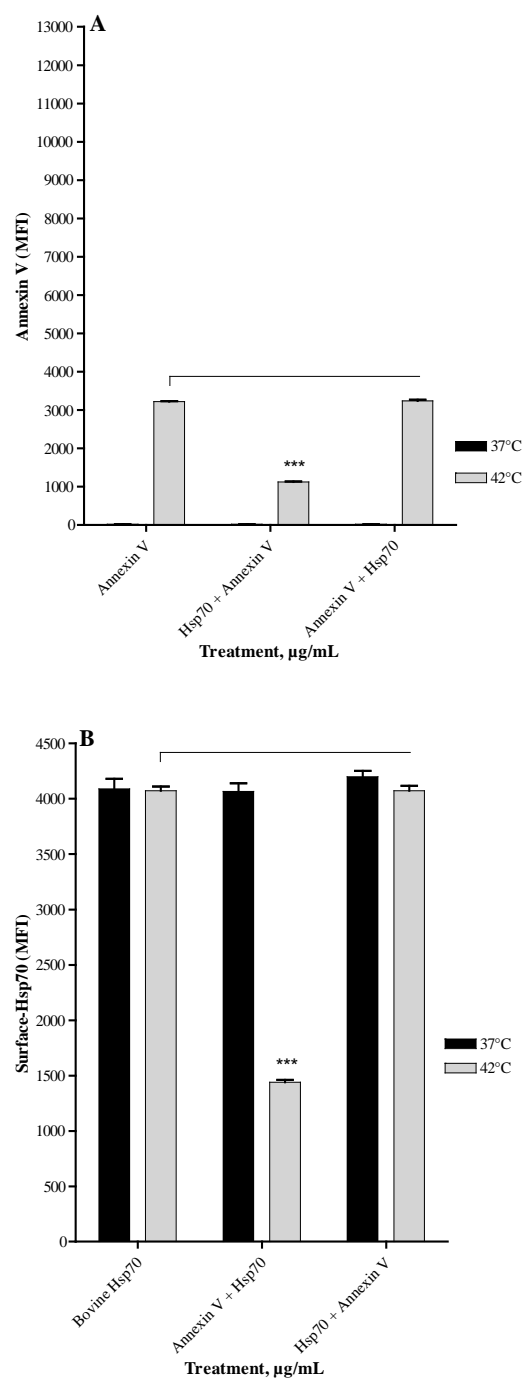
Either: Human leukocytes were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C, following pre-incubation with Annexin V for 20 minutes at 37°C. Or human leukocytes were incubated with Annexin V for 20 minutes at 37°C, following pre-incubation with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C. **(A)** Annexin V surface binding. **(B)** Hsp70 surface binding. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from Annexin V or Hsp70 control between mean treatments through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\*\* ( $P<0.001$ ).



**Figure 4.29: Binding of bovine Hsp70 to PS on the surface of lymphocytes at 37°C and in response to a 1 hour 42°C heat shock treatment.**

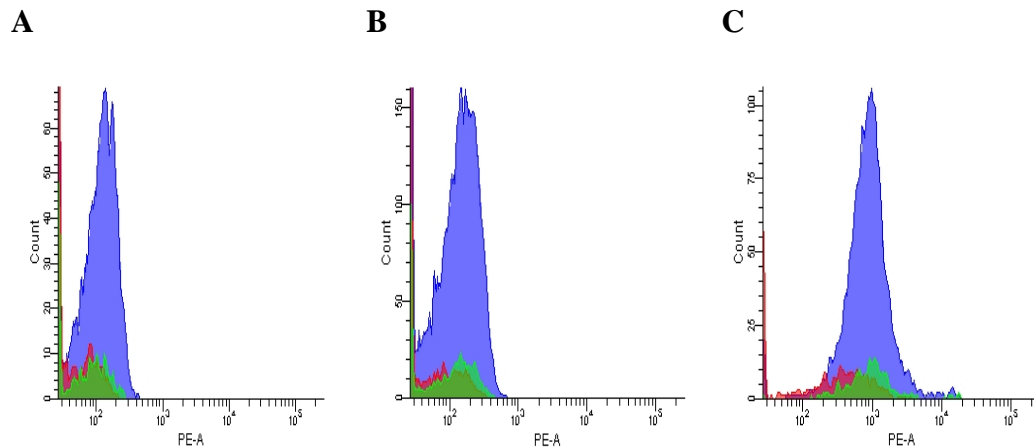
Either: Human leukocytes were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C, following pre-incubation with Annexin V for 20 minutes at 37°C. Or human leukocytes were incubated with Annexin V for 20 minutes at 37°C, following pre-incubation with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C. **(A)** Annexin V surface binding. **(B)** Hsp70 surface binding. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from Annexin V or Hsp70 control between mean treatments through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\*\* ( $P<0.001$ ).





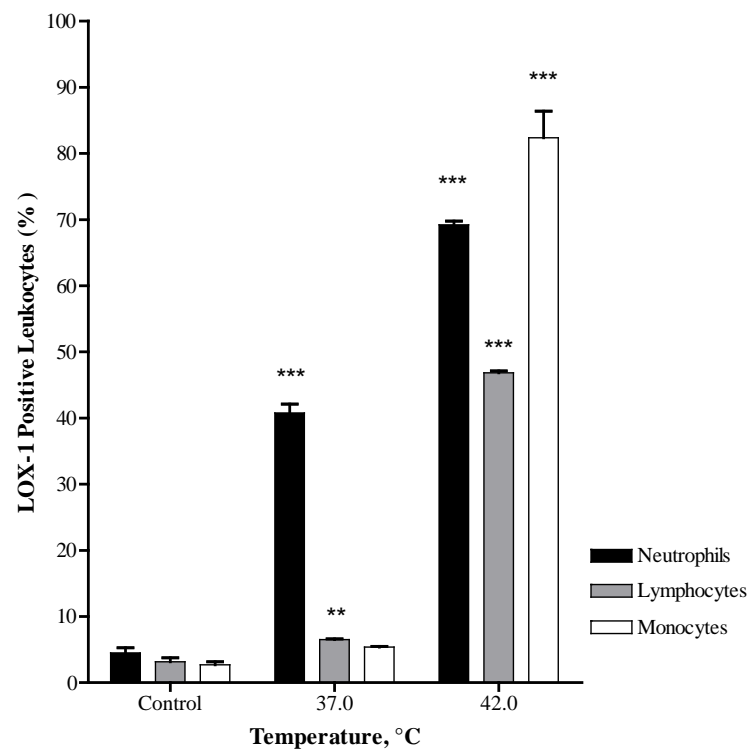
**Figure 4.30: Binding of bovine Hsp70 to PS on the surface of monocytes at 37°C and in response to a 1 hour 42°C heat shock treatment.**

Either: Human leukocytes were incubated with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C, following pre-incubation with Annexin V for 20 minutes at 37°C. Or human leukocytes were incubated with Annexin V for 20 minutes at 37°C, following pre-incubation with 10  $\mu\text{g/mL}$  bovine Hsp70 for 1 hour at 37°C. **(A)** Annexin V surface binding. **(B)** Hsp70 surface binding. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from Annexin V or Hsp70 control between mean treatments through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\*\* ( $P<0.001$ ).



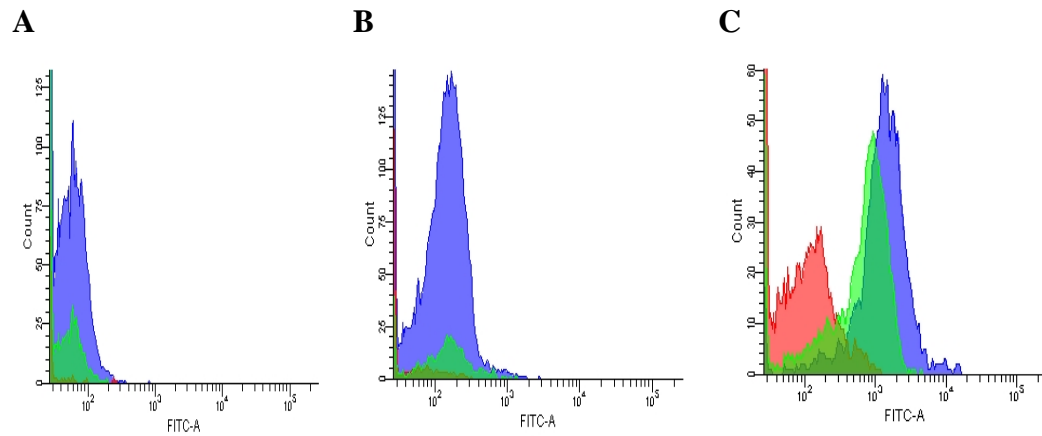
**Figure 4.31: LOX-1 analysis of human leukocytes using flow cytometry.**

Leukocytes were gated using FSC/SSC and antibodies to surface CD markers respectively. (A) No stain control sample, (B) LOX-1 expression at 37°C and (C) LOX-1 expression at 42°C. Neutrophils (■), lymphocytes (■) and monocytes (■). Data are representative of three independent experiments and 10,000 events were recorded.



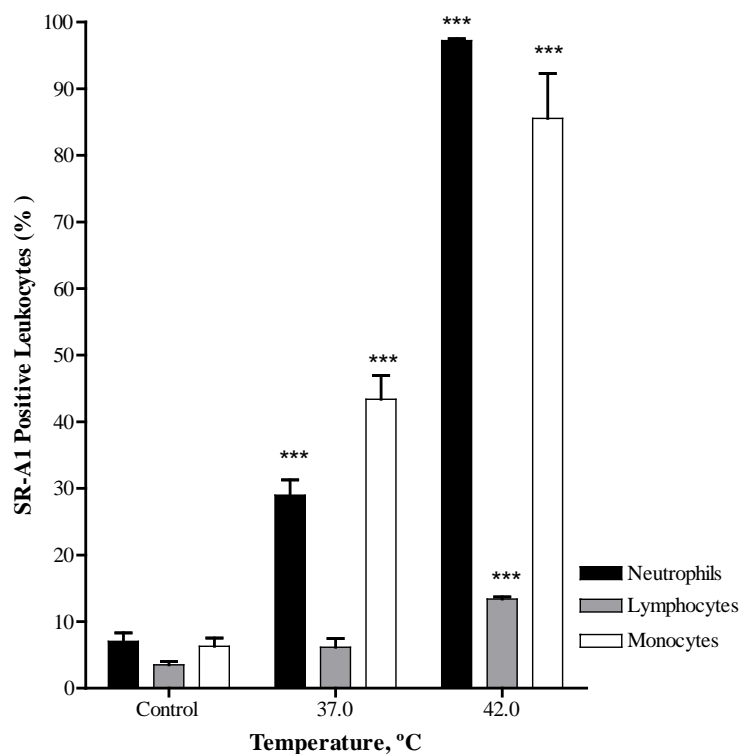
**Figure 4.32: Expression of LOX-1 on human leukocytes at 37°C and in response to 1 hour 42°C heat shock treatment.**

Neutrophils (■), lymphocytes (■) and monocytes (□). Data are presented as mean  $\pm$  SEM,  $n=3$ . Control = no stain control sample. Significance shown as difference from no stain control between mean treatments through use of one-way ANOVA with Bonferroni's multiple comparison *pot hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



**Figure 4.33: SR-A1 analysis of human leukocytes using flow cytometry.**

Leukocytes were gated using FSC/SSC and antibodies to surface CD markers respectively. (A) No stain control sample, (B) SR-A1 expression at 37°C and (C) SR-A1 expression at 42°C. Neutrophils (■), lymphocytes (■) and monocytes (■). Data are representative of three independent experiments and 10,000 events were recorded.



**Figure 4.34: Expression of SR-A1 on human leukocytes at 37°C and in response to 1 hour 42°C heat shock treatment.**

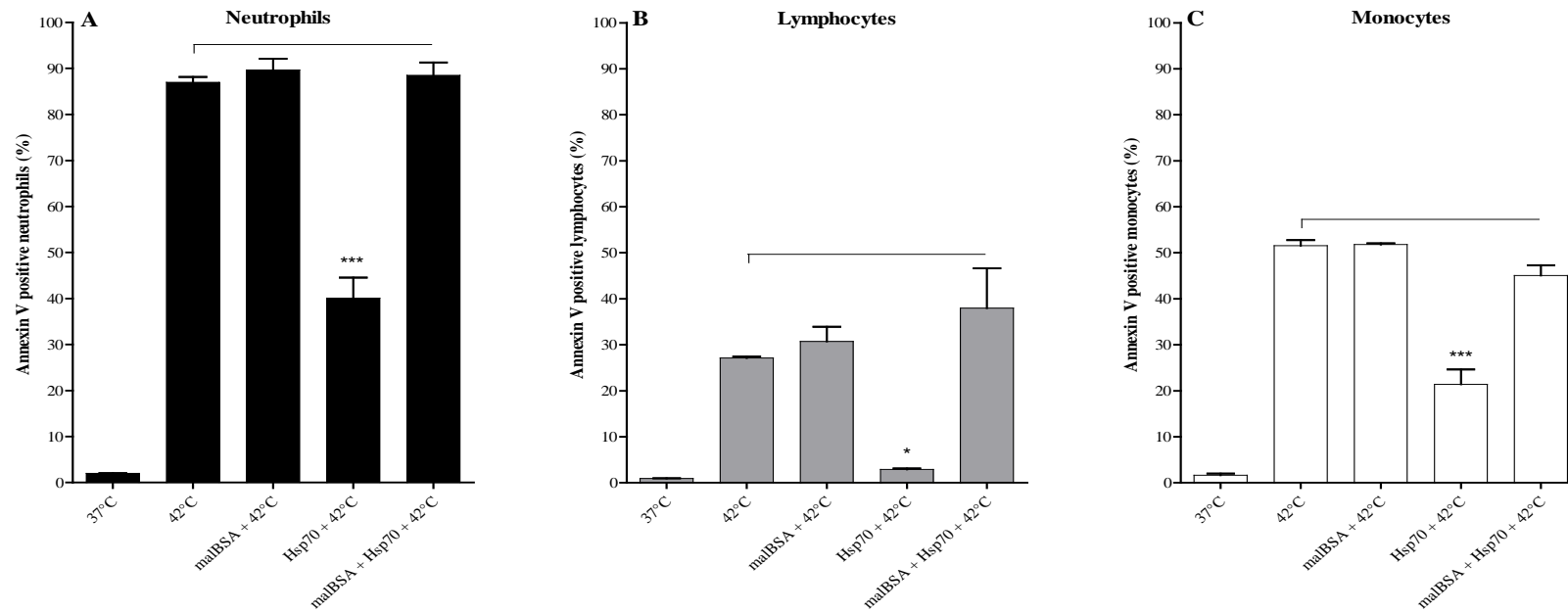
Neutrophils (■), lymphocytes (■) and monocytes (□). Data are presented as mean  $\pm$  SEM,  $n=3$ . Control = no stain control sample. Significance shown as difference from no stain control between mean treatments through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).

#### **4.3.7 Confirming the interaction between bovine Hsp70 and SRs does lead to protection from elevated temperature stress.**

Apoptosis and necrosis of human leukocytes in response to elevated temperatures following treatments with malBSA, fucoidan or poly (I) and/or extracellular bovine Hsp70 are presented in Figures 4.35-4.40.

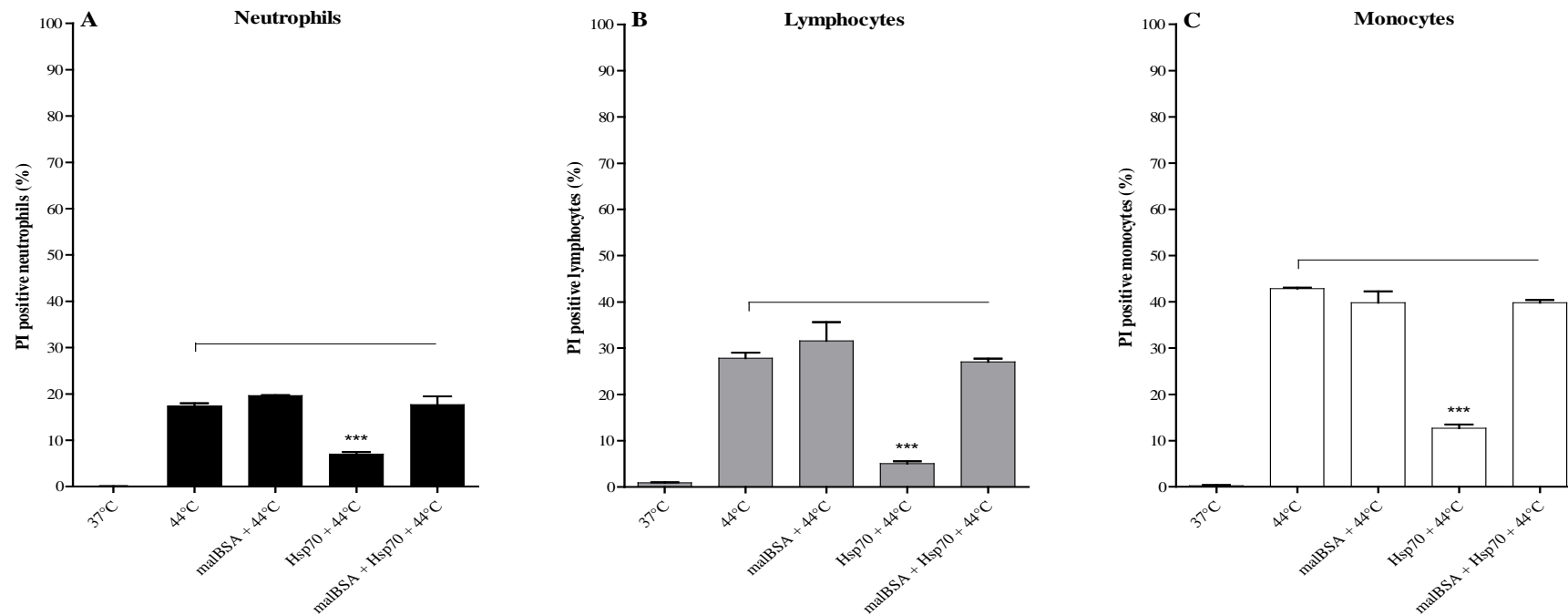
To confirm that the addition of extracellular bovine Hsp70 (10 µg/mL) to human leukocytes for two hours at 37°C, must bind to SRs on the surface of these cells in order to exert its cyto-protective functions under conditions of heat stress, competitive inhibition experiments were performed. Human leukocytes were either pre-incubated with 10 µg/mL of malBSA (Figures 4.35-4.36), fucoidan (Figures 4.37-4.38) or poly (I) (Figures 4.39-4.40) for up to six hours at 37°C alone or in combination with 10 µg/mL bovine Hsp70 for two hours at 37°C, followed by exposure to a two hour heat shock treatment at 42°C or 44°C. Human leukocytes incubated with bovine Hsp70 alone for two hours at 37°C prior to heat shock treatment led to significantly less ( $P<0.05$  to  $P<0.001$ ) apoptotic leukocytes compared to 42°C control cells (Figures 4.35, 4.37, 4.39), and also significantly less ( $P<0.01$  to  $P<0.001$ ) necrotic leukocytes compared to 44°C control cells (Figure 4.36, 4.38, 4.40). In contrast, human leukocytes exposed to malBSA, fucoidan or poly (I) alone for up to six hours at 37°C did not protect human leukocytes from the effects of elevated temperature stress (Figures 4.35-4.40). Furthermore, the pre-incubation of human leukocytes with malBSA, fucoidan or poly (I) for up to six hours at 37°C, prior to incubation with bovine Hsp70 led to a significant suppression of the protective effects exerted by bovine Hsp70 in response to heat shock at 42°C ( $P<0.01$  to  $P<0.001$ , Figures 4.35, 4.37, 4.39) or 44°C ( $P<0.001$ , Figures 4.36, 4.38, 4.40) compared to treatment with bovine Hsp70 alone. MalBSA significantly suppressed the protective effects of bovine Hsp70 in response to a 42°C heat shock in neutrophils, from 40 % to 88 % apoptotic cells ( $P<0.001$ , Figure 4.35A), in lymphocytes from 3 % to 38 % apoptotic cells ( $P<0.01$ , Figure 3.35B) and in monocytes from 21 % to 45 % apoptotic cells ( $P<0.001$ , Figure 4.35C) compared to control (Figure 4.35A-C). In addition, malBSA also significantly suppressed the protective effects of bovine Hsp70 in response to a 44°C heat shock, in neutrophils from 7 % to 18 % necrotic cells ( $P<0.001$ , Figure 4.36A), in

lymphocytes from 5 % to 27 % necrotic cells ( $P<0.001$ , Figure 3.36B) and in monocytes from 13 % to 40 % necrotic cells ( $P<0.001$ , Figure 4.36C) compared to control cells (Figure 4.36A-C). Similar to malBSA, fucoidan also significantly reduced the protective effects of bovine Hsp70 in response to a 42°C heat shock in neutrophils from 39 % to 86 % apoptotic cells ( $P<0.01$ , Figure 4.37A), in lymphocytes from 5 % to 30 % apoptotic cells ( $P<0.001$ , Figure 4.37B) and in monocytes from 21 % to 52 % apoptotic cells ( $P<0.001$ , Figure 4.37C) compared to control cells (Figures 4.37A-C). Furthermore, fucoidan also significantly suppressed the protective effects of bovine Hsp70 in response to a 44°C heat shock in neutrophils from 4 % to 16 % necrotic cells ( $P<0.001$ , Figure 4.38A), in lymphocytes from 8 % to 30 % necrotic cells ( $P<0.001$ , Figure 4.38B) and in monocytes from 15 % to 41 % necrotic cells ( $P<0.001$ , Figure 4.38C) compared to control cells (Figures 4.38A-C). In a similar action to that of malBSA and fucoidan, poly (I) also significantly reduced the protective effects of extracellular bovine Hsp70 in response to a 42°C heat shock in neutrophils from 45 % to 91 % apoptotic cells ( $P<0.001$ , Figure 4.39A), in lymphocytes from 9 % to 31 % apoptotic cells ( $P<0.001$ , Figure 4.39B) and in monocytes from 24 % to 52 % apoptotic cells ( $P<0.001$ , Figure 4.39C) compared to control (Figures 4.39A-C). In addition, poly (I) also significantly suppressed the protective effects of bovine Hsp70 in response to a 44°C heat shock in neutrophils from 9 % to 20 % necrotic cells ( $P<0.01$ , Figure 4.40A), in lymphocytes from 8 % to 29 % necrotic cells ( $P<0.001$ , Figure 4.40B) and in monocytes from 13 % to 40 % necrotic cells ( $P<0.001$ , Figure 4.40C) compared to control (Figures 4.40A-C).

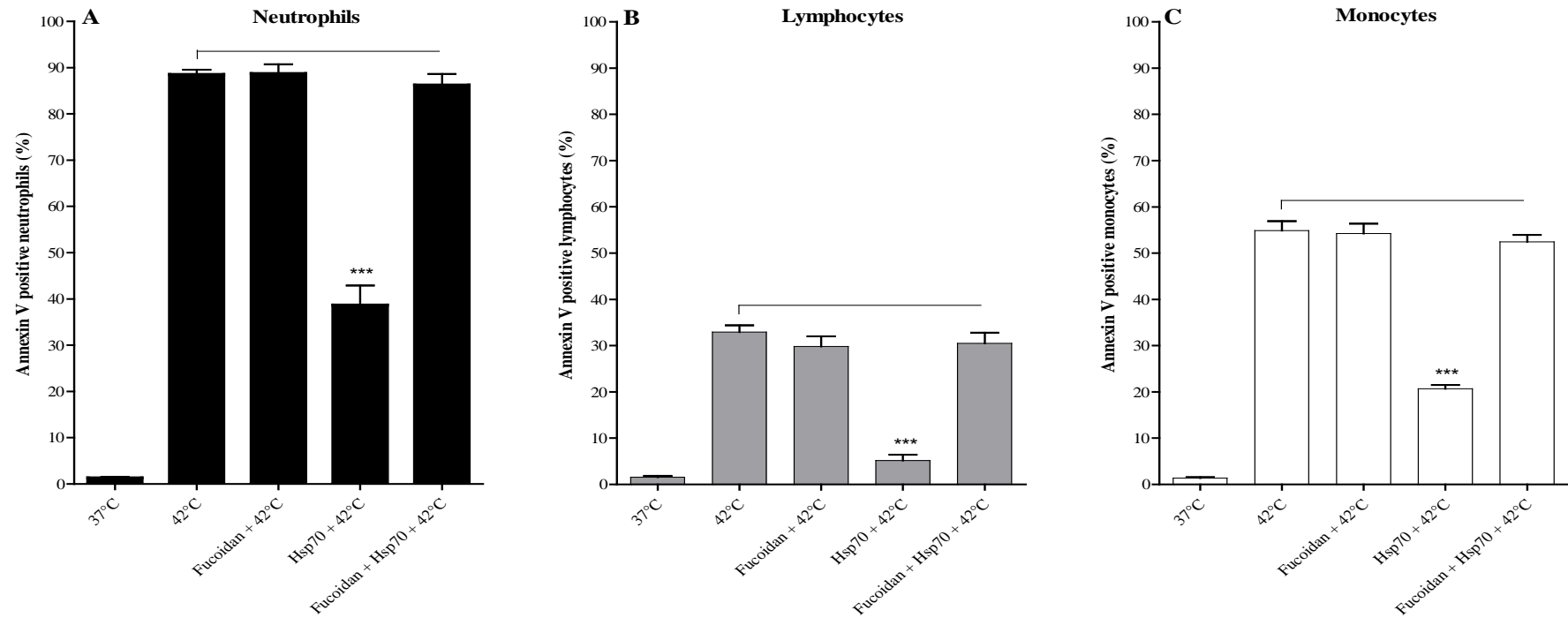


**Figure 4.35: Percentage of Annexin V positive human leukocytes in response to a 2 hour heat shock treatment at 42°C following a 6 hour pre-incubation with 10 µg/mL malBSA and/or a 2 h pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 42°C between mean treatments through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\*\* (P<0.001).



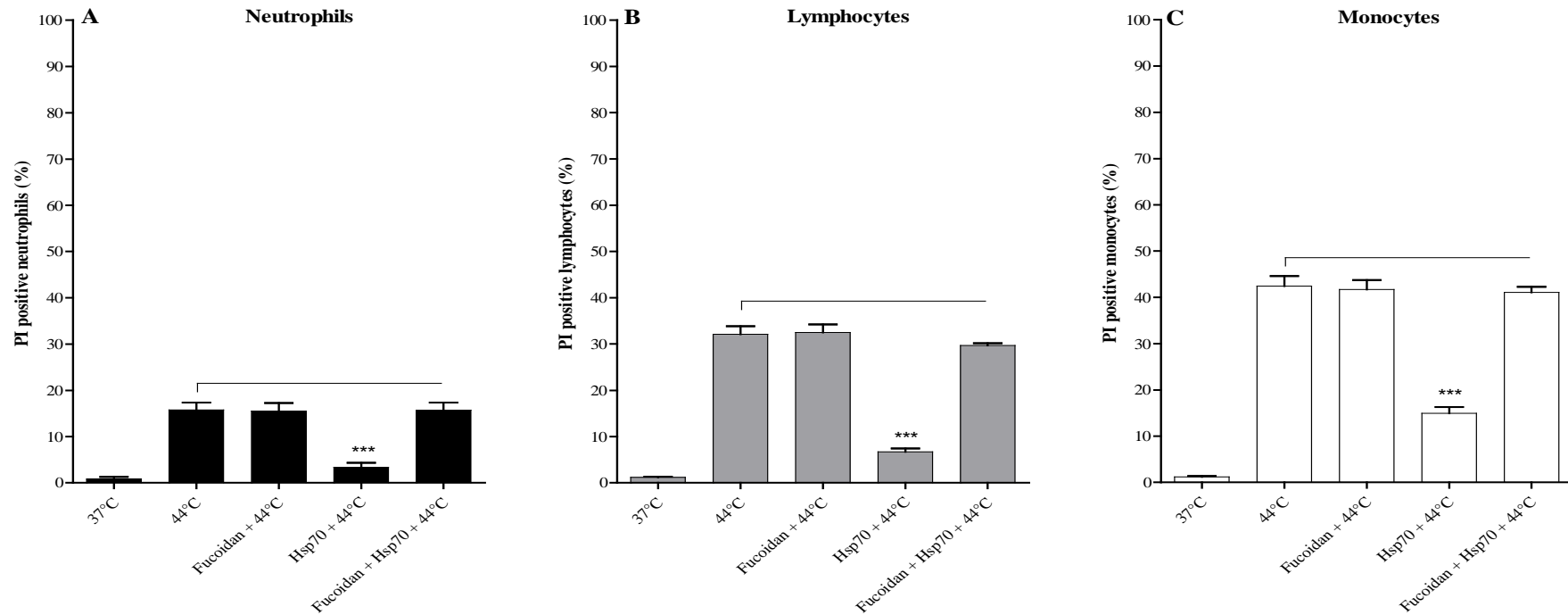
**Figure 4.36: Percentage of PI positive human leukocytes in response to a 2 hour heat shock treatment at 44°C following a 6 h hour pre-incubation with 10 µg/mL malBSA and/or a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.** Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean ± SEM, n=3. Significance shown as difference from 44°C between mean treatments through use of one-way ANOVA with Bonferroni's *post hoc* test, \*\*\* (P<0.001).



**Figure 4.37: Percentage of Annexin V positive human leukocytes in response to a 2 hour heat shock treatment at 42°C following a 6 hour pre-incubation with 10 µg/mL fucoidan and/or a 2 h pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

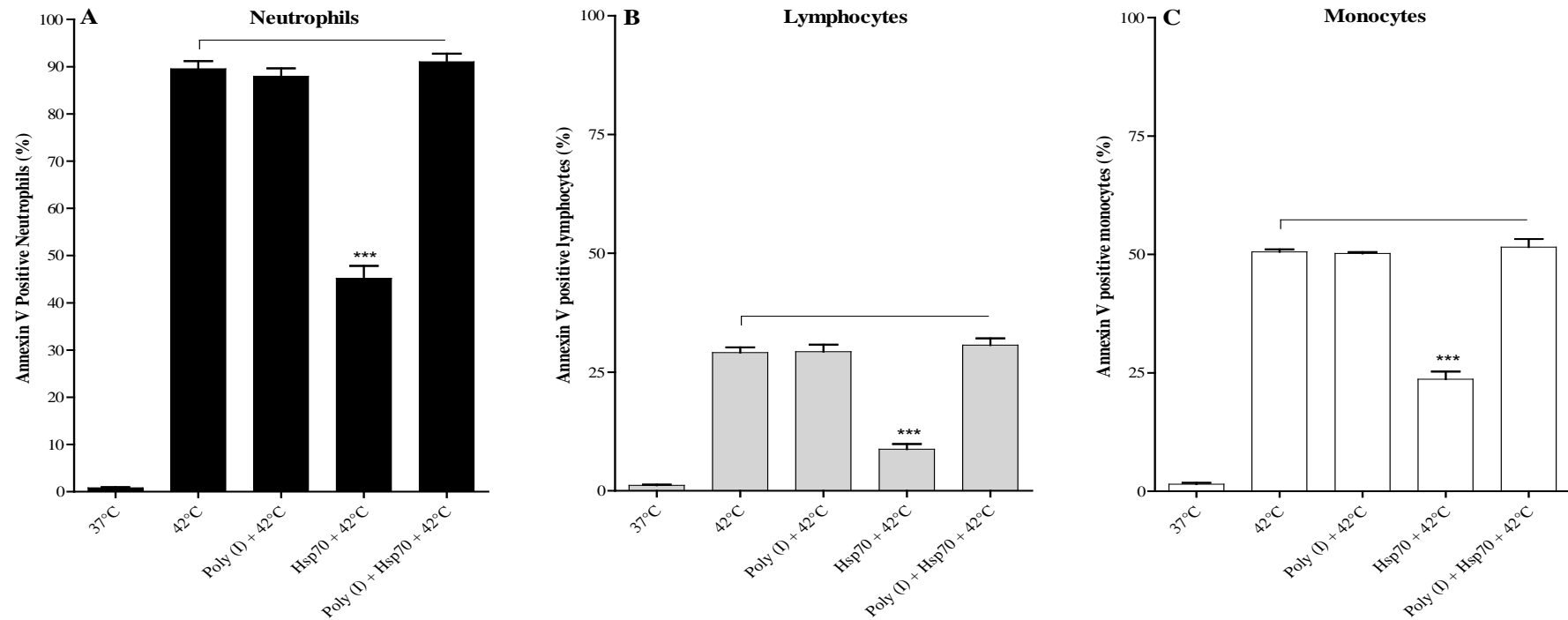
Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 42°C between mean treatments through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).



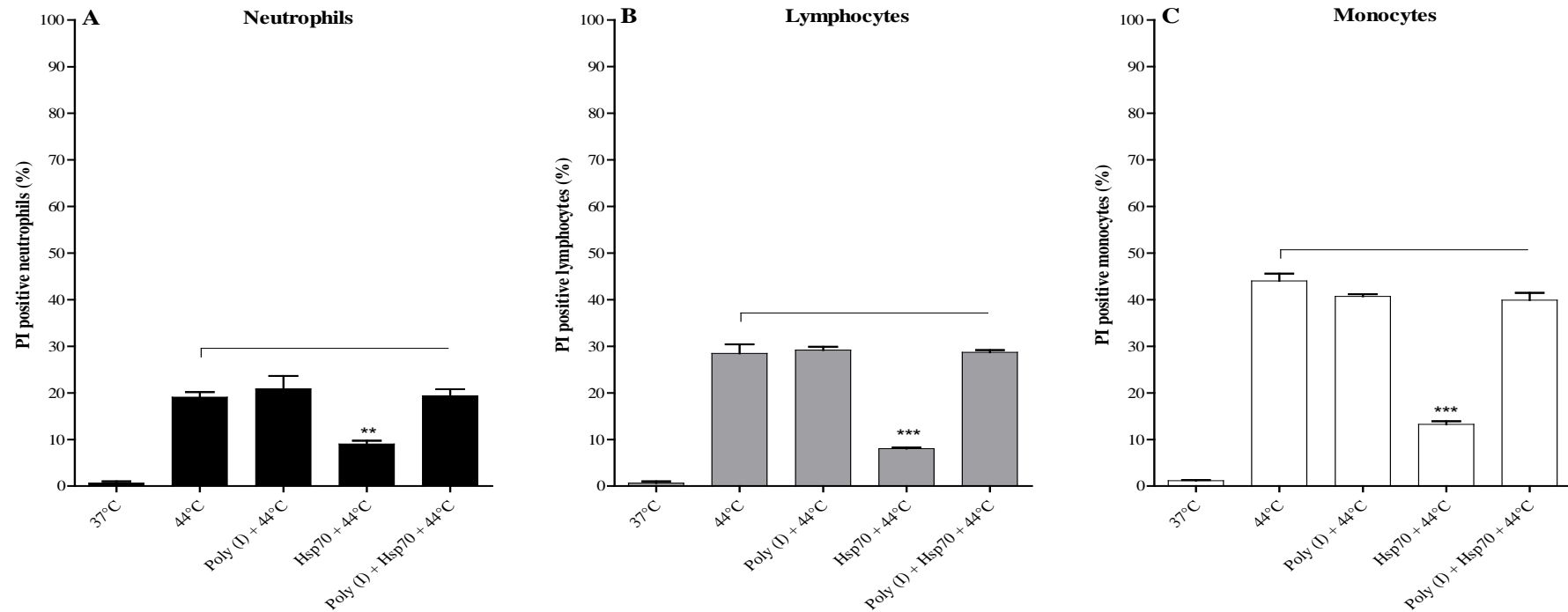


**Figure 4.38: Percentage of PI positive human leukocytes in response to a 2 hour heat shock treatment at 44°C following a 6 h hour pre-incubation with 10 µg/mL fucoidan and/or a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 44°C between mean treatments through use of one-way ANOVA with Bonferroni's *post hoc* test, \*\*\* (P<0.001).



**Figure 4.39: Percentage of Annexin V positive human leukocytes in response to a 2 hour heat shock treatment at 42°C following a 6 hour pre-incubation with 10 µg/mL poly (I) and/or a 2 h pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.** Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean ± SEM, n=3. Significance shown as difference from 42°C between mean treatments through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).



**Figure 4.40: Percentage of PI positive human leukocytes in response to a 2 hour heat shock treatment at 44°C following a 6 h hour pre-incubation with 10 µg/mL poly (I) and/or a 2 hour pre-incubation with 10 µg/mL bovine Hsp70 at 37°C.**

Neutrophils (A), lymphocytes (B) and monocytes (C). Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference from 44°C between mean treatments through use of one-way ANOVA with Bonferroni's *post hoc* test, \*\* (P<0.01), \*\*\* (P<0.001).

## **4.4 Discussion**

The aims of this chapter were to investigate the effects of extracellular bovine Hsp70 or recombinant human Hsp72 on apoptosis, and/or necrosis in response to elevated temperature stress in U937 monocytes and human leukocytes, and to determine possible cell surface interactions by which the HSPs could elicit these effects.

### **4.4.1 Binding and internalisation of extracellular HSPs in different cell types.**

The first part of the study was performed in order to determine interactions, including binding and internalisation, of extracellular bovine Hsp70 or human Hsp72 introduced into a cultured and primary cell line. The rationale for this study follows from the observations that HSPs when found in the extracellular milieu can influence physiological functions (Hightower & Guidon, 1989; Johnson & Tytell, 1993), including cyto-protection (Johnson et al. 1995; Houenou et al. 1996; Guzhova et al. 1998; Fujihara & Nadler, 1999; Guzhova et al. 2001; Kelty et al. 2002), through a possible interaction with a receptor on the cell surface (Asea et al. 2000b; Binder et al. 2000; Basu et al. 2001; Vabulas et al. 2002; Delneste et al. 2002; Thériault et al. 2005). Extracellular bovine Hsp70 or human Hsp72 were applied to U937 cells and human leukocytes at a concentration of 0-50 µg/mL for up to four hours. It was found that these proteins, but not a BSA control protein (data not shown), bind (in non-permeabilized cells) and internalise (permeabilized cells) in both cell types in a concentration and time-dependent manner. The data shows binding of extracellular HSPs to the cell surface of non-permeabilized U937 cells and human leukocytes took less than one hour as determined by flow cytometry and confirmed by fluorescence microscopy. Specifically, these proteins were observed bound to the cell surface of non-permeabilized U937 cells within twenty minutes of incubation at a concentration of 5 µg/mL human Hsp72 or 10 µg/mL bovine Hsp70. These proteins were also thought to be taken up by permeabilized U937 cells and readily internalised within forty minutes where a plateau was reached, leading to a significant increased amount of intracellular HSP compared to the values of non-permeabilized cells. These results are similar to previous findings by other groups (Negulyaev et al. 1996; Guzhova et al.

1998). Furthermore, the binding of extracellular HSPs whether human Hsp72 or bovine Hsp70 was also observed on non-permeabilized human leukocytes, but after a longer length of incubation compared to U937 cells, within forty minutes, but at a lower concentration of 5  $\mu\text{g/mL}$  for both neutrophils and monocytes and at a concentration of 10  $\mu\text{g/mL}$  for lymphocytes. The concentrations required to induce the responses seen in this study have been previously shown to be present in the circulation (0.1-5  $\mu\text{g/mL}$ ) and provides evidence for a potential functional relevance *in vivo* (Pockley et al. 1998; Rea et al. 2001; Njemini et al. 2003). These results are similar to previous findings (Alder et al. 1990; Johnson & Tytell, 1993; Arispe & De Maio, 2000; Vega et al. 2008). One of the first observations of HSP interaction with cell membranes came from Alder et al. (1990) who found human Hsp70 could interact with artificial lipid bilayers to form pores and ion-conducting channels in artificial bio-membranes. This research was followed by Negulyaev et al. (1996) who established that HSP/Hsc70 preparations could induce specific  $\text{K}^+$  channels on the U937 cell surface, furthermore Arispe & De Maio (2000) found Hsp70 to form cation-conducting channels in liposomes and more recently Vega et al. (2008) and Schilling et al. (2009) have demonstrated Hsp70 inserted into the plasma membrane after stress. The interaction of Hsp70 with the plasma membrane suggests a regulatory role for HSPs in cell adhesion, transport, receptor expression and internalisation (Vega et al. 2010). In addition, at this time point (forty minutes) a significant uptake and internalisation of these proteins were also detectable in all permeabilized leukocyte subtypes, increasing their total internal HSP content significantly compared to non-permeabilized cells. These results are in agreement with previous findings, including Fujihara and Nadler (1999) who observed Hsp70 uptake in human monocytes. Others have shown Hsp70 to be internalised and readily imported into both cytoplasmic and nuclear compartments of U937 monocytes (Guzhova et al. 1998), retinal ganglion cells (Fujihara & Nadler, 1999), neuroblastoma cells (Guzhova et al. 2001; Yu et al. 2001; Novoselova et al. 2005) and HUVECs (Pockley et al. 2009). Despite the evidence of increased levels of HSPs in permeabilized cells it cannot be completely concluded that the extracellular HSPs were internalised, it can also be speculated that the addition of extracellular HSPs induced intracellular *HSP* gene transcription and translation. This would need to be confirmed in future studies.

However this is highly unlikely because the converse has shown to be the case; intracellular Hsp70 expression is decreased by excess intracellular Hsp70 as it sequesters any free HSF1 (Gething & Sambrook, 1992; Morimoto, Sarge & Abravaya, 1992).

The binding of extracellular human Hsp72 in both cell types was always significantly higher than those seen with bovine Hsp70 and appears to be positively correlated with the rate of internalisation: U937 cells ( $r^2 = 0.9782$ ) and human leukocytes ( $r^2 = 0.9423$  neutrophils,  $r^2 = 0.9005$  lymphocytes and  $r^2 = 0.8769$  monocytes). However, the responses were always similar for both proteins and therefore it seemed appropriate to use bovine Hsp70 for the remainder of the experiments in this study due to cost implications.

#### **4.4.2 Extracellular bovine Hsp70 induced protection in different cell types.**

The data in the second part of this study demonstrated a protective effect of extracellular bovine Hsp70 on U937 cells and human leukocytes induced to apoptotic and necrotic cell death with elevated temperature stress, which supports the theory of Hsp70-mediated cell protection against factors inducing cell death (Guzhova et al. 1998). The data indicates a dose-dependent protection induced by application of 10 µg/mL bovine Hsp70 to U937 cells and human leukocytes for two hours prior to elevated temperature stress, which reduced apoptotic and necrotic cell death significantly. These data suggest extracellular bovine Hsp70 to have a functional role in the protection of cells either their membranes or intracellular components from *in vitro* elevated temperature stress. These results are similar to those observed in previous studies, including nutrient deprivation in cultured smooth muscle cells (Johnson & Tytell, 1993), axotomy of the sciatic nerve (Houenou et al. 1996), TNF- $\alpha$  induced apoptosis or necrosis of cultured U937 cells (Guzhova et al. 1998), trophic factor withdrawal in cultured motor neurons (Tidwell et al. 2004), hyperthermic stress and treatment with staurosporine in cultured neuroblastoma cells (Guzhova et al. 2001), rat retinal photoreceptors from light-induced cell death (Yu et al. 2001), a medullary slice preparation from neonatal rat from heat shock and excess of glutamate (Kelty et al. 2002) and induced endotoxin (LPS) tolerance in THP-1 cells (Aneja et al. 2006). Despite the differences in the cell types and stressors used in these studies, one important characteristic remained the same, the mode of cell death.

This study is one of the first to report the protection of cells from temperature induced necrosis with extracellular bovine Hsp70. Indeed, apart from Guzhova et al. (1998), there appears to be little literature reporting the protection of cells from necrotic cell death with extracellular HSPs, although there are several reports of inducible intracellular HSPs conferring protection against necrosis. These include, cultured neurones from glutamate toxicity (Rordorf et al. 1991), ischaemic neuronal injury (Kumar et al. 1995), ischaemic-induced tissue necrosis (Harder et al. 2005), and rat chondrocytes from experimental osteoarthritis (Grossin et al. 2006).

The protection of both cells types in this study from apoptotic or necrotic cell death using extracellular bovine Hsp70, as determined in the previous section is thought to be through cell binding and/or internalisation. Indeed, when extracellular HSPs were applied to both cell types there was an association between surface-bound HSPs and significantly less apoptotic cells, a positive correlation between surface-bound HSPs and significantly less necrotic U937 cells ( $r^2 = 0.9600$ ), and also evidence of an association between surface-bound HSPs and significantly less necrotic human leukocytes. Furthermore, the results indicated an association between internalised HSPs and apoptotic and necrotic U937 cells and human leukocytes as well as a positive correlation between internalised HSPs and significantly less apoptotic human monocytes ( $r^2 = 0.9041$ ), which suggests extracellular HSPs are capable of protecting cells from the effects of heat shock through possibly blocking or interfering with the events leading to cell death. Previous reports have suggested intracellular Hsp70 to interrupt the sequence of events in apoptotic cell death (Samali & Orrenius, 1998; Vayssier & Polla, 1998), through suppressing cell death-specific components such as Apaf-1, the release of cytochrome *c* or the processing of caspase-3 (Li et al. 2000; Mosser et al. 2000; Saleh et al. 2000; Matsumori et al. 2005). However, both Ran et al. (2004) and Robinson et al. (2005) claim extracellular Hsp70 promotes survival of cells by a mechanism other than directly interacting with cell death-specific components. The results presented in this study revealed extracellular bovine Hsp70 to reduce the appearance of PS and caspase-3 activity in both cell types, in addition to preventing necrosis. These results suggest extracellular HSPs when released from viable or necrotic cells, promote the survival of cells, either the cell it was released from (if viable),

or neighbouring cells, by acting upstream of caspase-3 activation or through endocytosis of cellular nutrients including calcium, glucose, iron or vitamins (A-C) essential for cellular viability, during times of physiological insult (Vega et al. 2010). It appears further work is required to elucidate the exact mechanism by which HSPs when present in the extracellular milieu prevent apoptotic or necrotic cell death.

Abundant levels of intracellular Hsp72 were detected in both U937 cells and human leukocytes as demonstrated in chapter three, taken together these results indicate that base line levels of intracellular Hsp70 are insufficient to maximally protect cells from the effects of elevated temperature stress. These results appear to be consistent with the theory of thermotolerance, where a mild heat shock elevates intracellular Hsp72 above base line levels which in turn reduces the extent of cell death after a subsequent severe stress (Wang & Borkan, 1996; Mosser et al. 2000). Therefore, extracellular HSPs may bind to and penetrate cells in an active form and play a compensatory role after stress to promote survival of cells, the maintenance of survival pathways or inhibit activation of cell death-specific events through apoptosis. On the other hand, extracellular HSPs binding to the cell surface through high-affinity receptor mediated processes may function to stabilise the plasma membrane, retaining membrane fluidity, the distribution of integral membrane proteins such as receptors and transporters (Carratù et al. 1996) and help protect cells from elevated temperature stress that would normally cause necrosis. Fujihara & Nadler (1999) suggested extracellular HSP uptake functions in cell-to-cell communication, in which the transfer of a protective response, in the form of HSPs from environmental stresses, is regulated through a promotion of intracellular transcription. This observation led to the hypotheses that *in vivo* exogenous HSPs may be released into the extracellular milieu from necrotic cells (Gallucci et al. 1999; Basu et al. 2000b; Berwin et al. 2001; Saito et al. 2005), where they may be taken up by neighbouring cells that are deficient in HSP and so are unable to mount a stress response and act as a stimulus to boost or strengthen the immune response under conditions of cellular stress (Guzhova et al. 2001). This will be discussed further in chapter five.



#### **4.4.3 Receptor mediated protection in different cell types.**

It is suggested that the protective activity of extracellular HSPs arises from their ability to interact with cell membranes through binding to receptors, altering their permeability characteristics leading to endocytosis. HSPs are able to bind to a number of receptors on the cell surface, including CD91 (Binder et al. 2000; Basu et al. 2001), CD14 (Asea et al. 2000b), CD40 (Becker et al. 2002) and LOX-1 (Delneste et al. 2002). In relation to the cells used in this study, it has been previously demonstrated that on monocytes TLRs facilitate the binding and internalisation of extracellular Hsp70 (Asea et al. 2000a), in addition to CD40 on T and B cells (Wang et al. 2001; Delneste et al. 2002). However, in this study PS and SRs were examined as potential receptor molecules for extracellular Hsp70 on both cell types under control and heat stressed conditions. HSPs, particularly members of the HSP70 family have been demonstrated to preferentially interact with several lipid components of the cell membrane (Gudion & Hightower, 1986; Wang et al. 2006), including the membrane lipid PS, followed by rapid incorporation into the lipid bilayer (Arispe et al. 2004; Vega et al. 2008; Schilling et al. 2009). However, PS should only be present on the surface of senescent cells and previous evidence suggests it is not detectable on the outer membrane leaflet in a normal population (Devaux, 1991), as the results of this study clearly demonstrated. Yet, in response to a pre-treatment with elevated temperature stress, PS was detectable on the outer membrane leaflet of both cell types and binding of bovine Hsp70 to both cell types was significantly suppressed by a pre-incubation with Annexin V. These results suggest PS as a potential binding site for extracellular bovine Hsp70 under conditions of elevated temperature stress, which are in agreement with previous studies (Arispe et al. 2004; Vega et al. 2008; Schilling et al. 2009). After binding of bovine Hsp70 to PS, an endocytosis mechanism may take place similar to that for Annexin V, which may facilitate the transport of extracellular HSPs from the outer membrane leaflet to the inside of the cell where it can perform cyto-protective functions.

Despite evidence of bovine Hsp70 binding to PS under conditions of stress, it is unlikely that PS serves as the interacting molecule for extracellular HSPs in normal conditions, thus in these circumstances binding may occur *via* a protein-protein interaction or be receptor mediated. It was found that Hsp70 binding to

both U937 cells and human leukocytes was suppressed but not completely abrogated by a pre-incubation with malBSA, a general SR ligand (Takata et al. 1989; Sakai et al. 1996) and two SR-A type SR ligands, thus suggesting the involvement of SRs in binding of extracellular bovine Hsp70. SR-A1 was detected on the surface of U937 cells at 37°C, which has not been previously demonstrated under control conditions. It may be speculated that the binding of extracellular bovine Hsp70 to SR-A1 is likely to cause its internalisation processing its removal from the circulation under control conditions. This protein through cell-to-cell signalling may then determine the outcome of the cell through either stimulating a pro- or anti-inflammatory immune response. On the other hand, there was no detection of LOX-1 which is in agreement with previous studies (Gingras et al. 2000; Berwin et al. 2003; Thériault et al. 2005). Furthermore, in response to treatment with elevated temperature stress there was a significant up-regulation of SR-A1 on the surface of U937 cells however no up-regulation of LOX-1. The up-regulation of SR-A1 implicates a role for this receptor in binding extracellular HSPs or HSP-protein complexes in response to environmental stress, facilitating their interactions with the plasma membrane where they may function to stabilise the membrane and induce the survival of the cell or induce receptor-mediated endocytosis and stimulate internal cytoprotection. The up-regulation of SR-A1 receptors on cells has been previously demonstrated. These include on monocytes and murine macrophages in response to M-CSF (Moulton et al. 1994; de Villiers et al. 1994), and on cultured smooth muscle cells and fibroblasts by phorbol ester (Pitas, 1990; Pitas et al. 1992), or cytokines including platelet-derived growth factor-BB (Inaba et al. 1992), however there are no previous reports of SR-A1 up-regulation on U937 cells in response to elevated temperature stress.

In contrast, LOX-1 was identified on neutrophils and to a lesser degree on lymphocytes and monocytes under control conditions which is not surprising since LOX-1 has been previously described as a tethering receptor for leukocyte homing supporting leukocyte adhesion and can mediate antigen processing and cross-presentation (Sawamura et al. 1997; Delneste et al. 2002; Hayashida et al. 2002). However, a previous report by Yoshida and co-workers suggests there is no evidence of LOX-1 detection on freshly isolated human monocytes, but was found on these cells after differentiation: mature human monocyte-derived

macrophages, suggesting LOX-1 as a marker of differentiation (Yoshida et al. 1998). In response to elevated temperature stress there was a significant up-regulation of LOX-1 on all three leukocyte subtypes. There are previous reports of an up-regulation of LOX-1 on cultured bovine aortic endothelial cells in response to pro-inflammatory stimuli including TNF- $\alpha$  or phorbol ester such as PMA (Sawamura et al. 1997; Kume et al. 1998), however there are no previous reports of LOX-1 up-regulation on human leukocytes in response to elevated temperature stress. In addition, SR-A1 was detected on the surface of neutrophils and to a lesser degree on lymphocytes as well as on monocytes, the latter of which is highly up-regulated during differentiation to macrophages, which will be addressed in chapter six. Additionally, in response to heat stress there was a significant up-regulation of SR-A1 on all three leukocyte subtypes. As mentioned there are previous reports of SR-A1 up-regulation (Pitas, 1990; Inaba et al. 1992; Pitas et al. 1992; de Villiers et al. 1994), however there are no previous reports of SR-A1 up-regulation on human leukocytes in response to elevated temperature stress. The up-regulation of LOX-1 and SR-A1 receptors in response to temperature stress can possibly lead to activation of the innate and adaptive immune response, which may increase phagocytosis of apoptotic cells as previously suggested (Vega & De Maio, 2005), roles established by neutrophils and macrophages. This will be addressed in chapter six. In addition, the up-regulation of these receptors also suggests a role for extracellular HSPs in facilitating the interactions of proteins or other cellular/chemical components with the extracellular membrane under conditions of stress, through the formation of HSP: protein complexes. This suggests a possible role for extracellular HSPs in facilitating removal of cellular debris. Furthermore, when human leukocytes were pre-treated with malBSA, fucoidan or poly (I) prior to administration of extracellular Hsp70, the survival-promoting activity of Hsp70 was significantly reduced in a dose-dependent manner. It is therefore proposed that bovine Hsp70 binds avidly to SR-A1 on U937 cells and SR-A1 and/or LOX-1 on human leukocytes, prior to internalisation under control and heat stressed conditions. This interaction was shown to be essential for cell protection against apoptotic and necrotic cell death. These results support the hypothesis that cells become more resistant to heat stress when their intracellular Hsp70 is increased through uptake of extracellular Hsp70.

## **Chapter 5**

### **The protection of human erythrocytes by extracellular Hsp72 through novel receptors.**

#### **5.1 Introduction**

Peripheral blood cells are constantly challenged by a variety of stressors such as oxidative stress, metabolic starvation, nutritional deficiencies and fever. The majority of these cells, such as leukocytes, have the ability to respond and tolerate the stress through the transcription of a novel set of genes (*HSP*) resulting in the production of intracellular HSPs, including the stress inducible Hsp72 (Tissieres et al. 1974; Parsell & Lindquist, 1993; Morimoto & Santoro, 1998; Lang et al. 2000; Ménoret et al. 2002). Mammalian erythrocytes however, derived from haemopoietic stem cells in bone marrow and which circulate in the blood for an average of 110-120 days (Klinken, 2002; Walsh et al. 2002), cannot up-regulate protective mechanisms because they are enucleate (discarding their nucleus during maturation), and therefore cannot synthesise proteins such as Hsp72 for protection (Klinken, 2002; Kabanova et al. 2009). This is unlike the nucleated erythrocytes of lower vertebrates including, the rainbow trout (Currie & Tufts, 1997), the brook trout (Lund, Lund & Tufts, 2003) and juvenile Atlantic salmon (Zarate & Bradley, 2003) which do respond to stress by synthesising Hsp72 (Currie & Tufts, 1997; Zarate & Bradley, 2003). Mammalian erythrocytes are therefore vulnerable to damage or detrimental effects from stressors such as elevated temperature, oxidative stress and metabolic starvation, disrupting erythrocyte function and delivery of oxygen to the tissues.

As determined in chapter three, there is increasing evidence for the presence of Hsp72 proteins on the cell surface, membrane-bound or embedded (Di Cesare et al. 1992; Chouchane et al. 1994; Multhoff et al. 1995; Kleinjung et al. 2003; Korbelik et al. 2005; Steiner et al. 2006; Gehrmann et al. 2008), and in the extracellular milieu (Tytell et al. 1986; Hightower & Guidon, 1989), where they have been proposed to offer an extracellular source of protection (Johnson & Tytell, 1993; Honenou et al. 1996; Guzhova et al. 1998; Fujihara & Nadler, 1999; Guzhova et al. 2001). Work in chapter four determined extracellular bovine Hsp70 to protect U937 cells and human leukocytes from heat shock

induced injury and as discussed, the protective effects of these extracellular stress proteins are thought to be mediated through cell surface receptors (Thériault et al. 2005; Thériault et al. 2006). These receptors include, SRs such as CD91 (Binder et al. 2000), CD36 (Armesilla et al. 1996), LOX-1 (Delneste et al. 2002) and SR-A (Berwin et al. 2003). It has previously been demonstrated that human erythrocytes express virus binding ligands (Ohyama et al. 1993; Ruvoen-Clouet et al. 1995) as well as blood group antigens (Ohyama et al. 1993), and to date there is only evidence of the SR CD36 on the cell surface of human erythrocytes (Handunnetti et al. 1992; van Schravendijk et al. 1992).

### **5.1.1 Aims**

The aims of this chapter are to determine whether extracellular bovine Hsp70 or recombinant human Hsp72 is able to protect human erythrocytes in response to osmotic stress, uric acid or an acute temperature stress *in vitro*, followed by *in vivo* and *ex vivo* experiments. Also, the possible cell surface interactions by which the HSPs can afford this protection is investigated.

## **5.2 Methods**

All preparations and cell culture experiments were performed using aseptic technique in a class II tissue culture hood.

### **5.2.1 Preparation of cells for experimental treatments.**

Human erythrocytes were isolated and prepared for treatment as described in section 2.3.11.

### **5.2.2 Preparation of HSPs for experimental treatments.**

HSPs used for experiments were: bovine Hsp70 or recombinant human Hsp72. Stock solutions used were 0.5 mg/mL in dH<sub>2</sub>O (Bovine Hsp70), and 0.1 mg/mL in PBS (Human Hsp72).

### **5.2.3 Time course experiments with bovine Hsp70 or human Hsp72.**

Two *in vitro* time course experiments were performed:

- 1) Bovine Hsp70 was applied to human erythrocytes at concentrations of 30, 20, 10, 5 and 0 µg/mL diluted in phenol red-free 10 % RPMI. Cells were then incubated at 37°C for up to 4 hours.
- 2) Bovine Hsp70 or human Hsp72 was applied to human erythrocytes at a concentration of 10 µg/mL diluted in phenol red-free 10 % RPMI. Cells were then incubated at 37°C for up to 90 minutes.

### **5.2.4 Pre-treatment of cells with bovine Hsp70 or human Hsp72.**

Bovine Hsp70 or human Hsp72 were made-up to concentrations of 50, 30, 25, 20, 10, 5, 1, 0.01, 0.1 or 0 µg/mL in phenol red-free 10 % RPMI before being applied to cells for 30 minutes or 1 hour at 37°C before further treatments.

### **5.2.5 Heat shock treatment of cells.**

Two *in vitro* heat treatment experiments were performed using:

- 1) A thermal gradient bar set to 37.7°C–46.2°C for up to 90 minutes or 46.2°C–52.1°C for 1 hour then transferred to a 37°C incubator for 1 hour recovery.
- 2) A temperature-controlled stirred water bath set to 41°C for up to 4 hours followed by 1 hour recovery at 37°C.

### **5.2.6 Measuring erythrocyte lysis.**

Erythrocyte lysis was measured by haemoglobin leakage as described in section 2.3.32.

### **5.2.6 Determination of erythrocyte fragility.**

Erythrocyte fragility test: when exposed to a hypotonic solution erythrocytes take in increasing quantities of water, swell until the capacity of their cell membrane is exceeded, and burst releasing haemoglobin. However, if they are exposed to a hypertonic solution, for example of NaCl, the erythrocytes lose intracellular fluid, shrink, and break up. The erythrocyte fragility test is based on the measure of the resistance of erythrocytes to haemolyse by osmotic stress, using decreasing strengths of hypotonic NaCl solutions, through measuring the degree of haemolysis colourimetrically at RT (Massaldi, Richieri & Mel, 1988).

Following treatments, erythrocytes were centrifuged (350 g, 10 minutes at 25°C) and supernatant was removed and discarded. NaCl was then applied to human erythrocytes at concentrations of 20, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 and 0 mM diluted in dH<sub>2</sub>O. Cells were incubated for 2 minutes at 25°C, centrifuged (350 g, 10 minutes at 25°C) and transferred to a 96-well non-binding plate. Erythrocyte lysis was measured as above.

### **5.2.8 Treatment of cells with uric acid.**

Uric acid at concentrations of 10, 5 and 1 µg/mL in phenol red-free 10 % RPMI were applied to cells for 2 minutes at 25°C then immediately centrifuged at 350 g for 10 minutes at 25°C. Erythrocyte lysis was then determined as described in section 2.3.32.

### **5.2.9 Measurement of cell surface and internalised HSPs.**

Flow cytometry and fluorescence microscopy were used to measure bovine Hsp70 or human Hsp72 on the cell surface (membrane-bound on non-permeabilized cells) and on/inside (permeabilized cells) human erythrocytes (Sections 2.3.20-2.3.21).

Human erythrocytes were incubated with an anti-Hsp72 FITC conjugated antibody for 40 minutes at 4°C in the dark.

- 1) Flow Cytometry: Anti-Hsp72: FITC was applied to erythrocytes at a 1:100 dilution (DPBS containing 5 % FBS) 50  $\mu$ L/sample.
- 2) Fluorescence Microscopy: Anti-Hsp72: FITC was applied to cells at a 1:100 dilution (DPBS containing 1 % BSA) 50  $\mu$ L/sample.

#### **5.2.10 Western blot detection of Hsp72 or SR-A1.**

Erythrocyte membranes were prepared for analysis using methods described in section 2.3.22. Hsp72 or SR-A1 were visualised following SDS-PAGE (section 2.3.24) and western blotting (section 2.3.26).

#### **5.2.11 *In vivo* heat treatment.**

Three *in vivo* heat treatment experiments were performed:

- 1) The hands of three healthy volunteers were immersed up to the wrist in a hot water bath at 42°C for 1 hour. Blood samples were taken from fingers prior to immersion, after heat shock treatment and 1 hour recovery.
- 2) The individual fingers of volunteers were heat shocked at different temperatures (37.7°C-49.6°C) for 1 hour using a thermal gradient bar. Blood samples were taken from fingers prior to immersion and after treatment.
- 3) The hands of volunteers were immersed up to the wrist in a hot water bath at 42°C for 1 hour. Blood samples were taken from different fingers every 10 minutes for up to 1 hour from the heat treated and hand maintained at RT.

#### **5.2.12 Detection of potential receptors for Hsp72.**

The presence of potential Hsp72 receptors on the cell surface of human erythrocyte was tested using two methods (Section 2.3.29):

- 1) Competitive inhibition experiments using: Annexin V for PS analysis (2.5  $\mu$ L/well diluted in 50  $\mu$ L of 1X binding buffer), anti-CD77 for Gb3 analysis (20  $\mu$ L/well) or receptor agonists malBSA, fucoidan, or poly (I) (100  $\mu$ L/well of 10  $\mu$ g/mL dilution in antibody wash buffer) at 37°C.
- 2) Receptor specific antibodies: anti-LOX-1 or anti-SR-A1 (100  $\mu$ L/well of a 1:10 or 1:50 dilution in antibody wash buffer) at 37°C.

For the indirect detection of LOX-1 and SR-A1, secondary R-PE or FITC conjugated IgG antibodies were used (100  $\mu$ L/well of a 1:10 or 1:50 dilution in antibody wash buffer) at 37°C.



### 5.3 Results

The stability or fragility of the human erythrocyte membrane was determined by exposure *in vitro* to elevated temperatures, osmotic shock or increased levels of uric acid. In each method, cell lysis, as determined by haemoglobin leakage was measured by absorbance at 550 nm.

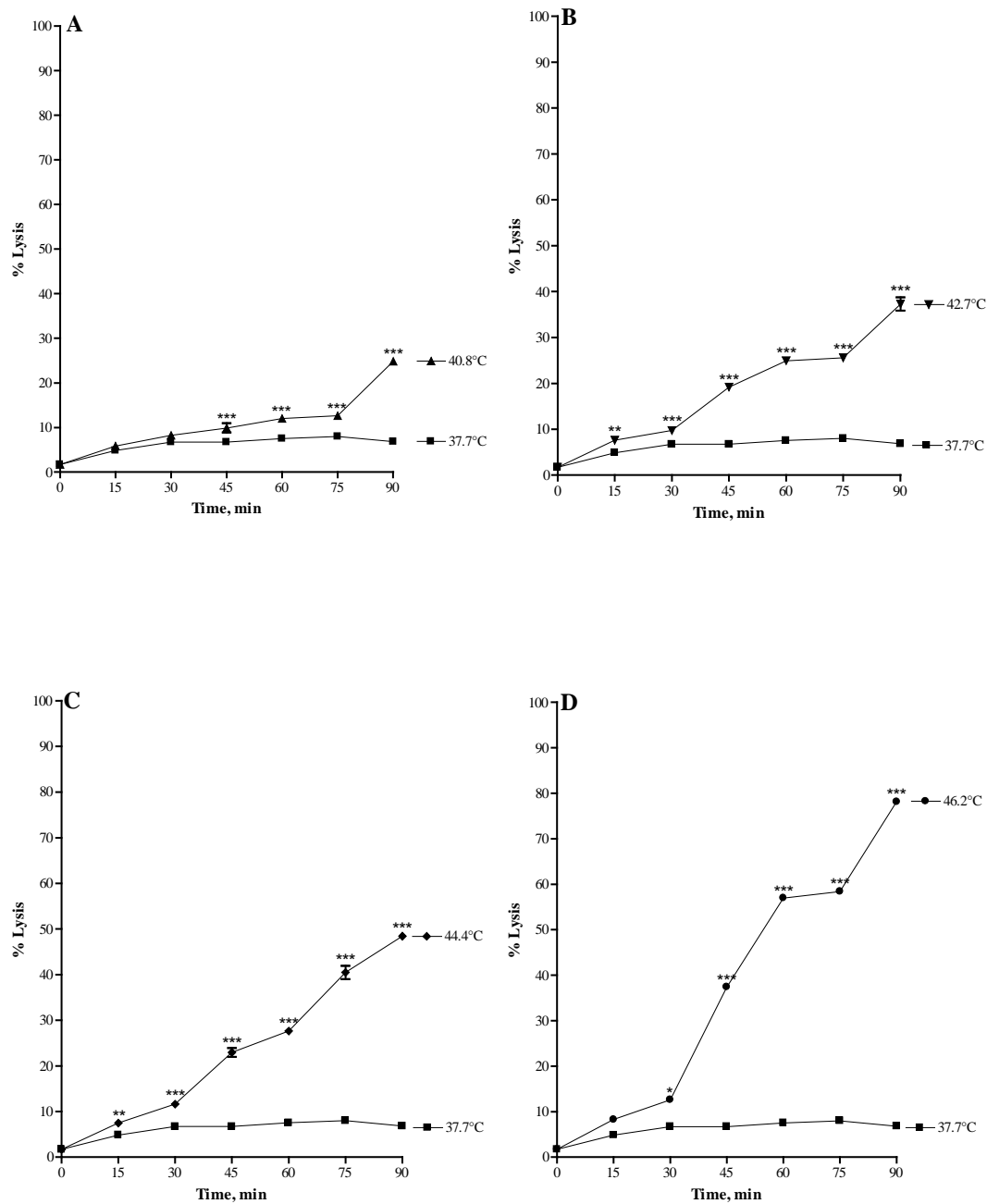
#### 5.3.1 Fragility of erythrocyte membranes following temperature stress as determined by erythrocyte lysis.

Erythrocyte lysis following the exposure of cells to elevated temperatures are presented in Figures 5.1-5.4.

Human erythrocytes were exposed to conditions of elevated temperature stress for ninety minutes, erythrocyte lysis was measured and percentage lysis was determined. The first heat treatment used in these initial experiments reflected fever, a condition characterised by an increase in internal body temperature to levels above normal and which is considered by physicians to be a protective body response to combat infection (Durack, 1997; Marinkovic et al. 2009). Treatment of human erythrocytes with the physiological temperature of 40.8°C, caused a significant increase of erythrocyte lysis after forty-five minutes of exposure ( $P<0.001$ , Figure 5.1A), which increased significantly with time, reaching approximately 30 % lysis after ninety minutes ( $P<0.001$ , Figure 5.1A). At temperatures above 40.8°C there was also a significant increase of erythrocyte lysis ( $P<0.01$ , Figure 5.1B-D), but which occurred within fifteen minutes of exposure (Figure 5.1B-D) and which increased in a time-dependent manner (Figure 5.1B-D), resulting with approximately 40 % (42.7°C), 50 % (44.4°C) and 70 % (46.2°C) lysis after ninety minutes of elevated temperature stress (Figure 5.1B-D). The addition of bovine Hsp70 (0-30  $\mu\text{g/mL}$ ) for up to four hours prior to heat treatment (1 hour, 46.2°C) resulted in a significant reduction in erythrocyte lysis ( $P<0.001$ , Figure 5.2). In fact, after just one hour of incubation with bovine Hsp70 a significant reduction (23 %) in erythrocyte lysis was seen compared to control ( $P<0.001$ , Figure 5.2), indicating that the erythrocytes were being protected. However, the lowest concentration of bovine Hsp70 tested (5  $\mu\text{g/mL}$ ) provided no protection (Figure 5.2). Increasing both the incubation time and concentration of bovine Hsp70 provided a greater and significant

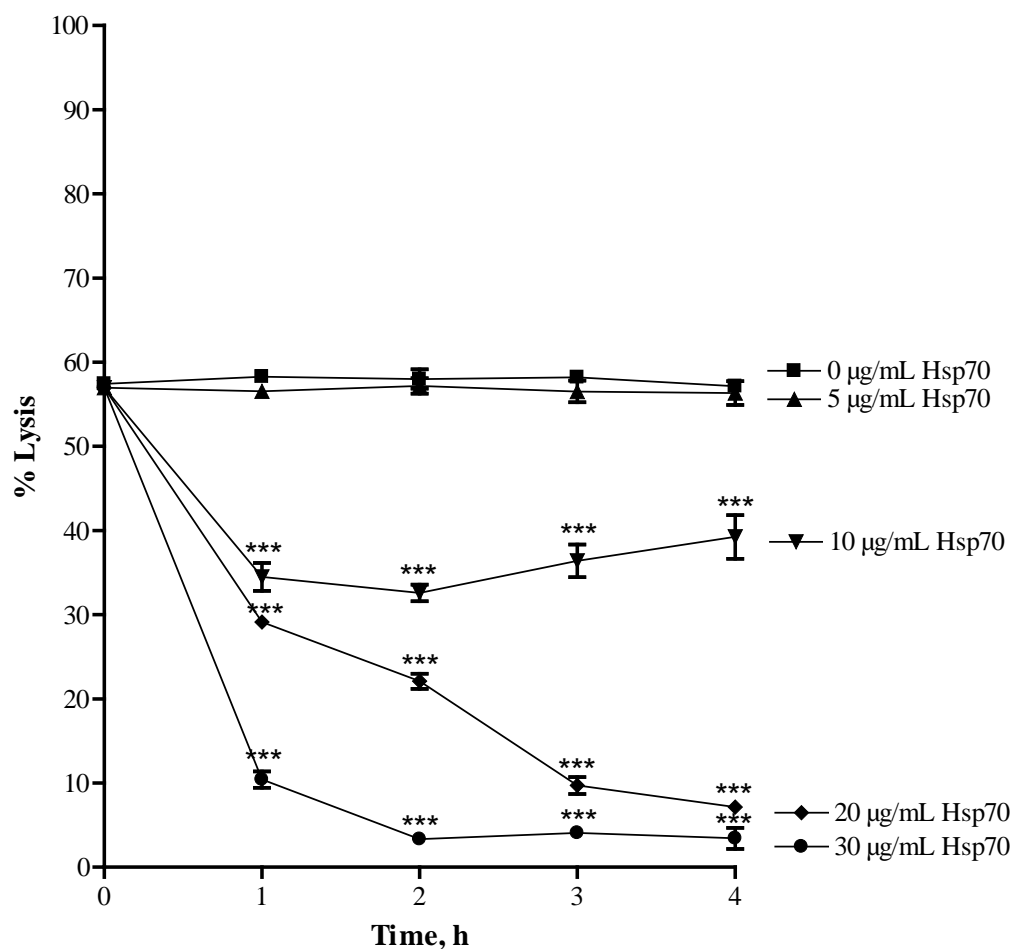
protection from the elevated temperature stress ( $P < 0.001$ , Figure 5.2). To further demonstrate the protective nature of extracellular bovine Hsp70, human erythrocytes were exposed to increasing temperatures of 46.2°C-52.1°C following a one hour pre-incubation with 0-30 µg/mL bovine Hsp70. Again, bovine Hsp70 caused a significant reduction of cell lysis ( $P < 0.001$ , Figure 5.3), and the protection observed increased with increasing concentrations of bovine Hsp70. Although these temperatures used in this experiment were not physiologically relevant, they were used simply to demonstrate protection by extracellularly administered bovine Hsp70.

Experiments were then performed at physiological temperatures: human erythrocytes were treated at 41°C for up to four hours. Erythrocyte lysis was again observed in controls at 41°C, though to a lesser extent than seen at the higher temperatures (Figure 5.4). This lysis was significantly reduced up to 60 % as a result of one hour pre-treatment with 0-10 µg/mL bovine Hsp70 ( $P < 0.001$ , Figure 5.4), showing bovine Hsp70 can protect erythrocytes from physiologically relevant temperature stress.



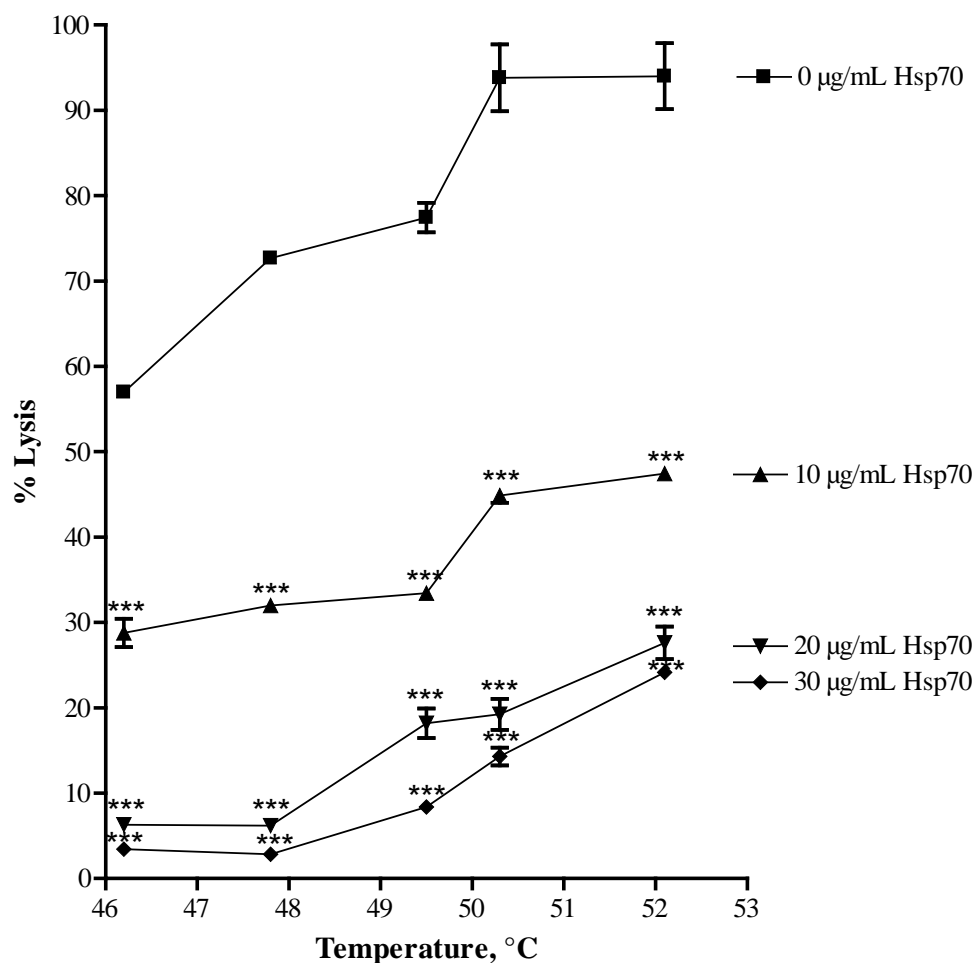
**Figure 5.1: Erythrocyte lysis in response to 0–90 minutes of elevated temperature stress at 40.8°C (A), 42.7°C (B), 44.4°C (C) or 46.2°C (D) and following 1 hour recovery at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . \* Indicates significant difference between mean time points compared to 37.7°C through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



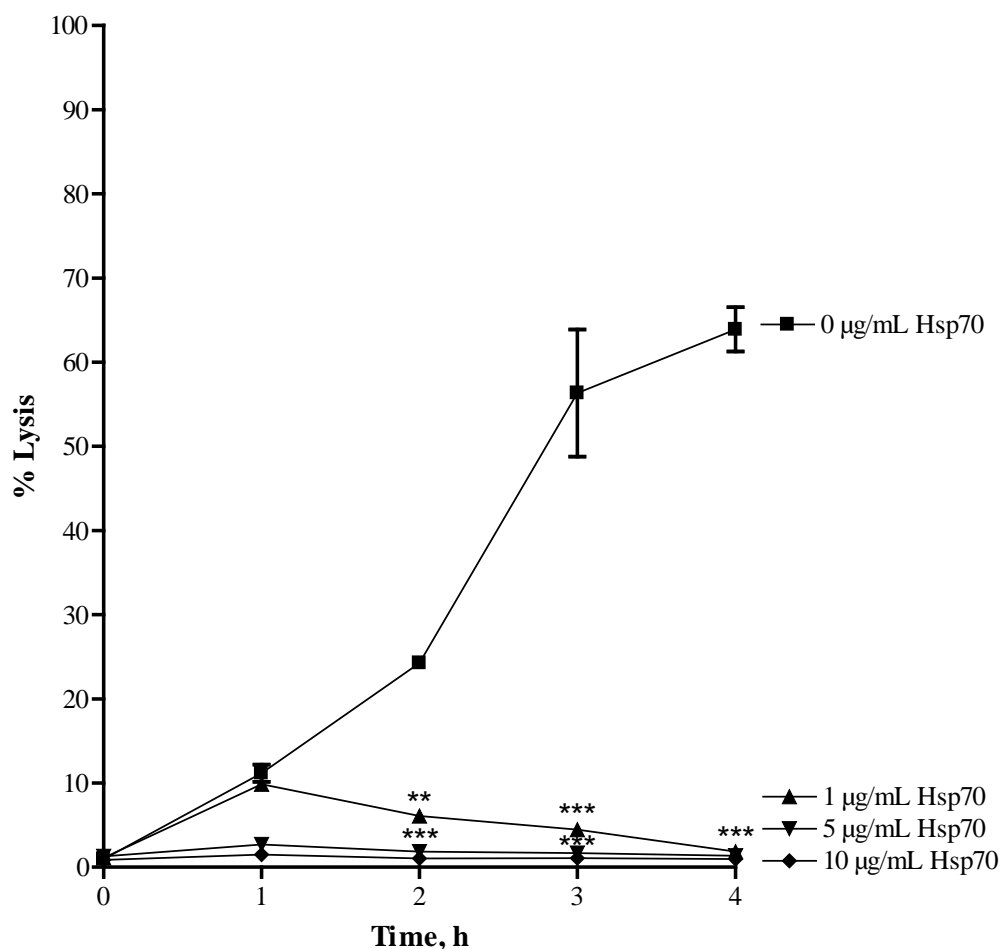
**Figure 5.2: Hsp70 reduces erythrocyte lysis in response to heat shock at 46.2°C.**

Erythrocytes were pre-treated with 0-30 µg/mL bovine Hsp70 for 0-4 hours at 37°C, followed by 1 hour heat shock treatment at 46.2°C. Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean time points compared to 0 µg/mL Hsp70 through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).



**Figure 5.3: Hsp70 reduces erythrocyte lysis in response to heat shock (46.2°C-52.1°C)**

Erythrocytes were pre-treated with 0-30 µg/mL bovine Hsp70 for 1 hour at 37°C, followed by 1 hour heat shock treatment. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean temperature points compared to 0 µg/mL Hsp70 through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



**Figure 5.4: Hsp70 prevents erythrocyte lysis in response to heat shock at 41°C.**

Erythrocytes were pre-treated with 0–10 µg/mL bovine Hsp70 for 1 hour at 37°C, followed by 0–4 hour heat shock treatment at 41°C. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean time points compared to 0 µg/mL Hsp70 through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).

### **5.3.2 Erythrocyte lysis following exposure to osmotic shock and increased levels of uric acid.**

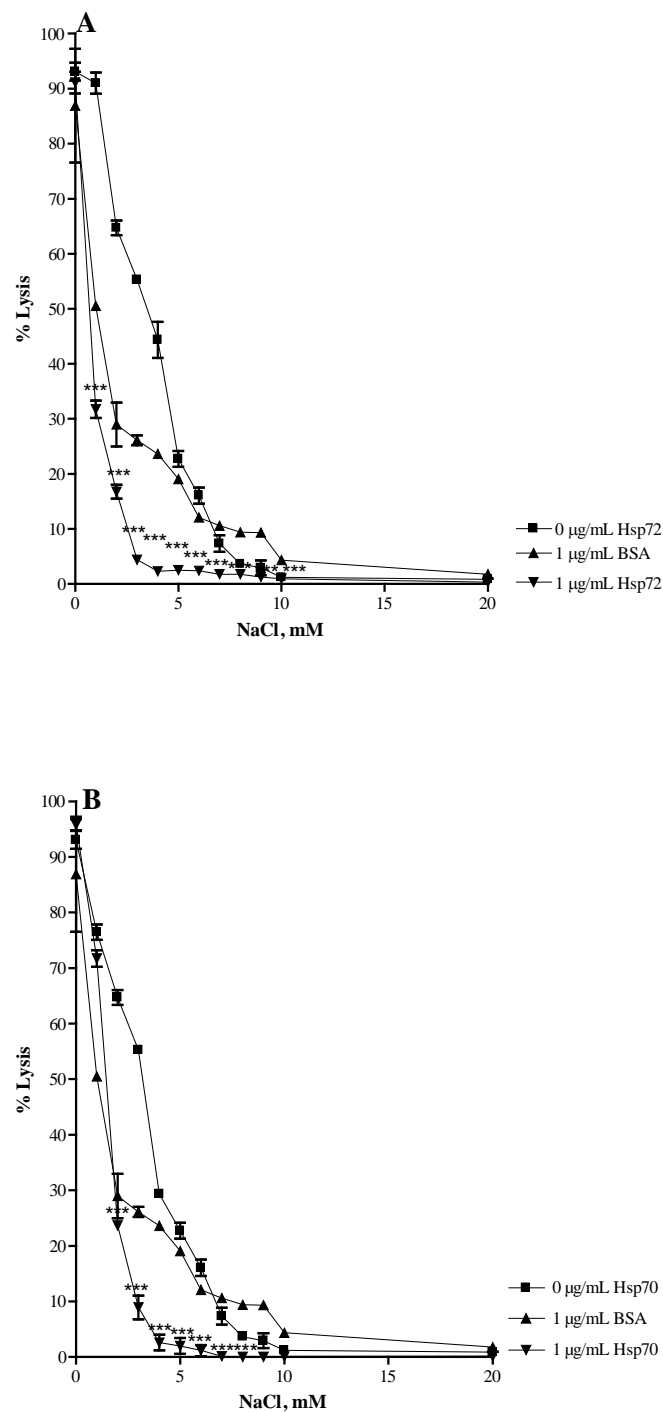
Erythrocyte lysis following treatments with osmotic shock and uric acid are presented in Figures 5.5-5.7.

Human erythrocytes were exposed to conditions of osmotic shock (hypotonic lysis) using decreasing concentrations of NaCl (0-20 mM) in order to determine erythrocyte membrane fragility *in vitro* (Bolton, 1949; Penha-Silva et al. 2007). Decreasing concentrations of NaCl below 9 mM caused a significant increase in erythrocyte lysis compared to isotonic concentrations ( $P < 0.01$ , Figure 5.5A-B). Pre-incubation of erythrocytes with 1  $\mu\text{g/mL}$  human Hsp72 (Figure 5.5A) or bovine Hsp70 (Figure 5.5B) prior to osmotic shock significantly reduced erythrocyte lysis compared to cells treated with 0-20 mM NaCl only ( $P < 0.001$ , Figure 5.5A-B). Pre-incubation with BSA had no effect on the fragility of the erythrocytes (Figure 5.5A-B). Incubation of erythrocytes in 3.5 mM NaCl resulted in rapid lysis of ~50 % cells (Figure 5.5), and this concentration was chosen to determine membrane fragility in subsequent experiments. Pre-incubation of erythrocytes with  $>0.5 \mu\text{g/mL}$  bovine Hsp70 or human Hsp72 completely prevented erythrocyte lysis when treated with 3.5 mM NaCl ( $P < 0.001$ , Figure 5.6). Pre-incubation with BSA had no effect on the fragility of the erythrocytes (Figure 5.6). Human Hsp72 was found to be more effective than bovine Hsp70 at protecting erythrocytes at concentrations  $<1 \mu\text{g/mL}$  ( $P < 0.001$ , Figures 5.5-5.6).

Uric acid, a potent pro-oxidant, was also used to determine erythrocyte membrane fragility. Normal levels of uric acid vary in human blood plasma for both sexes ranging from 2.0-7.0 mg/dL for females to 3.0–8.0 mg/dL for males (Rowlett, 2001). Abnormally high levels of uric acid (hyperuricemia) are particularly toxic to both human tissues and cells (Rowlett, 2001). Incubation of human erythrocytes isolated from venous (Figure 5.7A) and finger prick (Figure 5.7B) blood for two minutes with  $>5.0 \mu\text{g/mL}$  uric acid caused a significant increase in erythrocyte lysis compared to control ( $P < 0.001$ , Figure 5.7). Significantly more lysis was observed in the erythrocytes isolated from venous blood compared to finger prick blood when incubated with  $>5.0 \mu\text{g/mL}$  uric acid

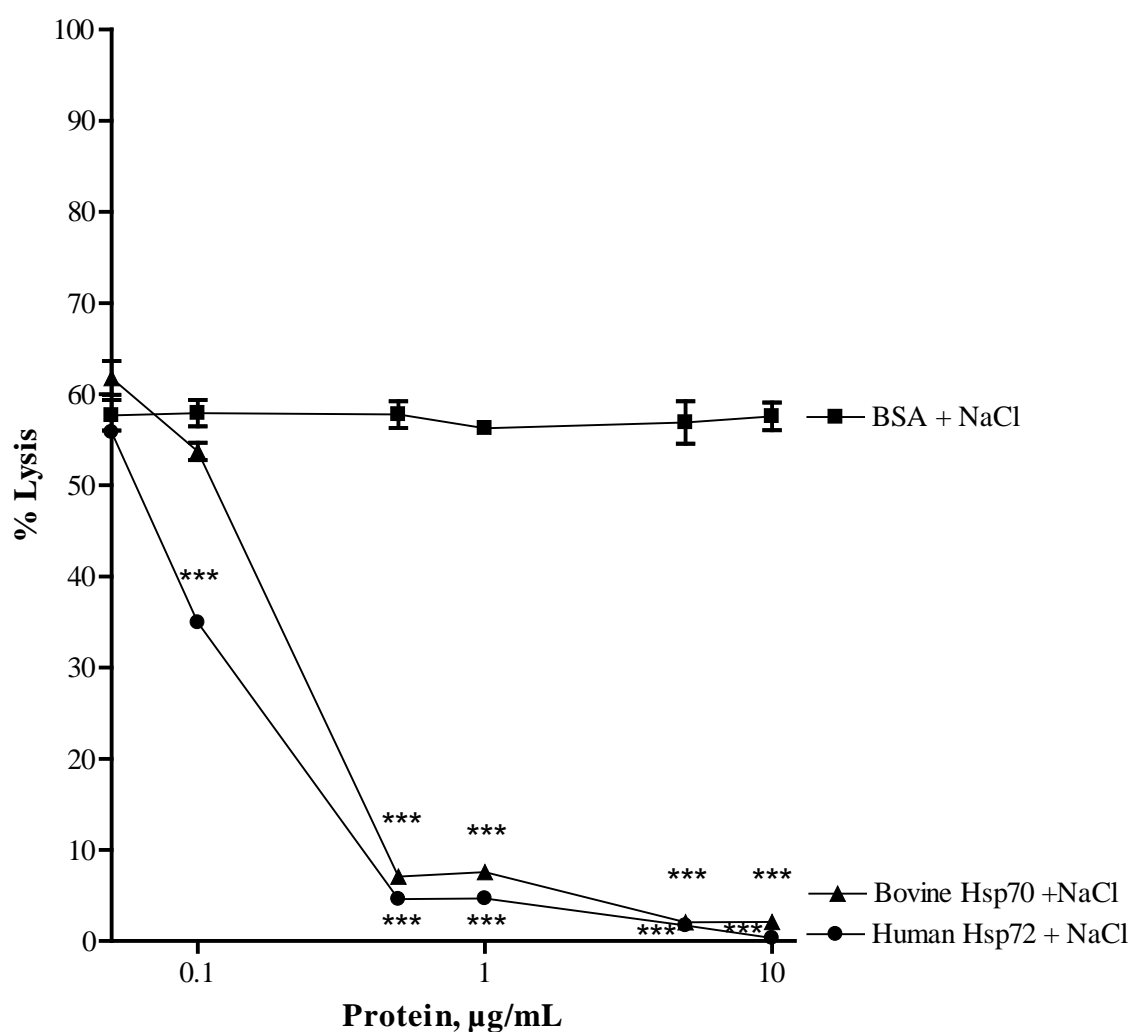
( $P < 0.01$ , Figure 5.7). Incubation of cells with 1  $\mu\text{g/mL}$  of bovine Hsp70 or human Hsp72 for one hour prior to exposure to increasing uric acid concentrations significantly reduced erythrocyte lysis ( $P < 0.001$ , Figure 5.7). Similar to treatments using osmotic stress, human Hsp72 was found to be more effective at providing erythrocyte protection compared to bovine Hsp70 ( $P < 0.001$ , Figure 5.7). These results indicate Hsp70 and Hsp72 can potentially protect erythrocytes *in vivo* from physiologically relevant stresses.





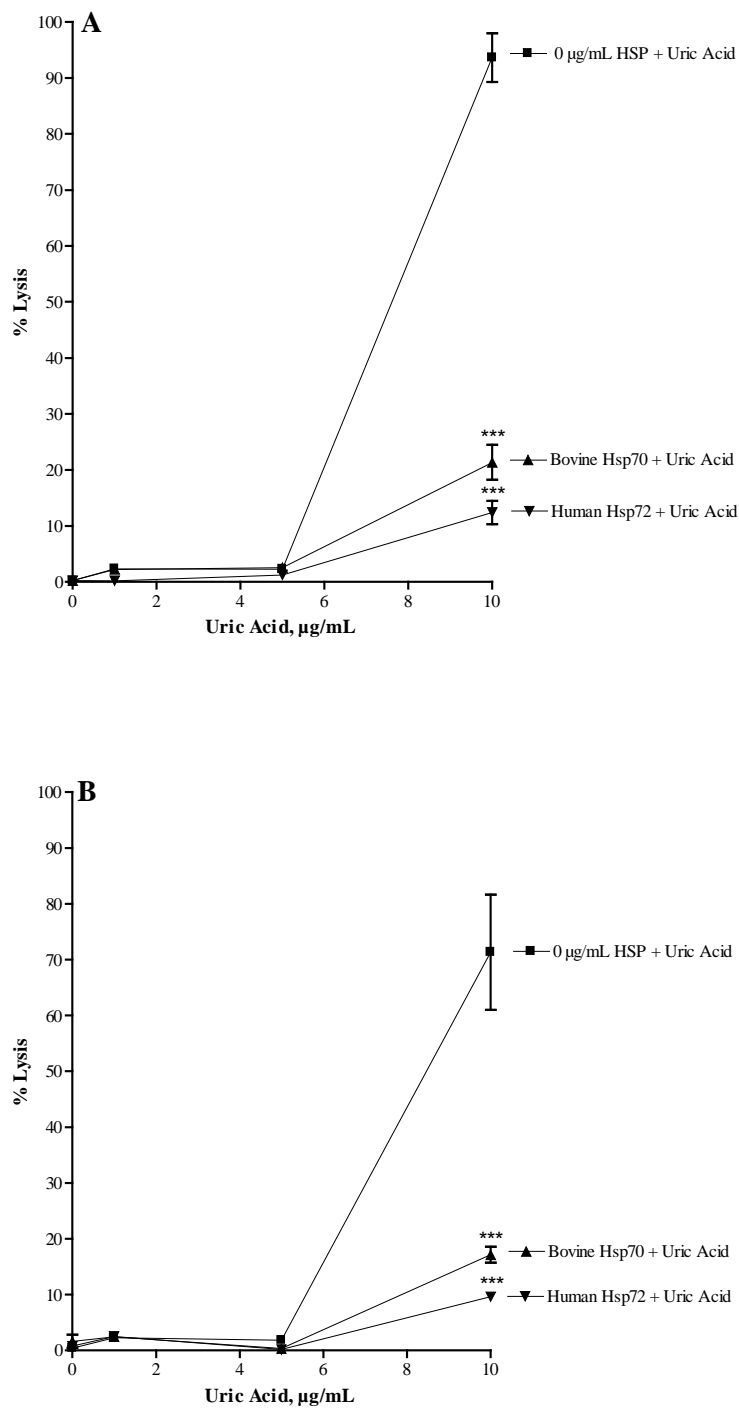
**Figure 5.5: Erythrocyte lysis in response to osmotic stress with 0-20 mM NaCl with or without a 1 hour pre-incubation with human Hsp72 (A) or bovine Hsp70 (B).**

Erythrocytes were pre-treated with 0 or 1  $\mu\text{g/mL}$  human Hsp72, bovine Hsp70 or BSA for 1 hour at  $37^\circ\text{C}$ , followed by NaCl treatment. Data are presented as mean  $\pm$  SEM,  $n=2$ . Significance shown as difference between mean concentration points compared to 0  $\mu\text{g/mL}$  Hsp70 through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



**Figure 5.6: Bovine Hsp70 and human Hsp72 decrease the fragility of human erythrocyte membranes.**

Erythrocytes were pre-treated with 0-10  $\mu\text{g/mL}$  bovine Hsp70, human Hsp72 or BSA for 1 hour at  $37^\circ\text{C}$  and subjected to osmotic stress with 3.5 mM NaCl. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean concentration points compared to BSA control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



**Figure 5.7: Bovine Hsp70 and human Hsp72 reduce erythrocyte lysis from cells isolated from venous (A) or finger prick (B) blood in response to uric acid treatment.**

Erythrocytes were pre-treated with 0 or 1  $\mu\text{g/mL}$  bovine Hsp70 or human Hsp72 for 1 hour at 37°C, followed by uric acid treatment (0-10  $\mu\text{g/mL}$ ). Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean concentration points compared to 0  $\mu\text{g/mL}$  HSP through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).

### 5.3.3 Detection of bovine Hsp70 or human Hsp72 in human erythrocytes

Surface binding (on non permeabilized cells) and internalisation (on/inside permeabilized cells) of HSPs in human erythrocytes following treatments with extracellular bovine Hsp70 or human Hsp72 are presented in Figures 5.8–5.12 and Table 5.1.

Exogenous human Hsp72 (10  $\mu\text{g/mL}$ ) was found to bind to the cell surface of non-permeabilized human erythrocytes (Figure 5.8A) and to internalise (in permeabilized cells) within twenty minutes of incubation at 37°C (Figure 5.9A), which increased up to sixty minutes. These data were confirmed by flow cytometry where binding of human Hsp72 to the erythrocyte surface ( $P<0.001$ , Figure 5.8B, Table 5.1) and internalisation ( $P<0.001$ , Figure 5.9B, Table 5.1) within one hour was observed. In addition, extracellular bovine Hsp70 was also detected on the surface of, and internal to, human erythrocytes within one hour of incubation ( $P<0.001$ , Table 5.1), however human Hsp72 was found to bind to the cell surface of erythrocytes and to internalise more readily than bovine Hsp70 at a concentration of 10  $\mu\text{g/mL}$  ( $P<0.001$ , Table 5.1).

A more detailed analysis of the surface binding (on non-permeabilized cells) and internalisation (on/in permeabilized cells) of extracellular human Hsp72 and bovine Hsp70 was then performed over a period of ninety minutes at 37°C. Following the incubation of human erythrocytes with extracellular HSPs (10  $\mu\text{g/mL}$ ) for twenty minutes at 37°C, a significant 20-fold increase ( $P<0.001$ ) in the binding of human Hsp72 (Figure 5.10A), and an almost 7-fold increase ( $P<0.001$ ) in the binding of bovine Hsp70 (Figure 5.10B) was observed compared to the 0  $\mu\text{g/mL}$  control. Furthermore, incubation of human erythrocytes with either extracellular human Hsp72 or bovine Hsp70 for twenty minutes also observed a significant increase of HSP internalisation. A 14-fold increase of internal HSP was observed with human Hsp72 ( $P<0.001$ , Figure 5.10A) and an almost 3-fold increase ( $P<0.01$ , Figure 5.10B) was observed with bovine Hsp70 compared to the 0  $\mu\text{g/mL}$  control. At incubation times lower than this (ten minutes) a non-significant detection of both surface and internalised Hsp72 or Hsp70 was observed compared to the 0  $\mu\text{g/mL}$  control (Figure 5.10A-B). Binding of both human Hsp72 and bovine Hsp70 to the erythrocyte surface

continued to significantly increase over time ( $P < 0.001$ , Figure 5.10A-B), until sixty minutes where a peak in binding was observed, a 42-fold increase ( $P < 0.001$ ) with human Hsp72 (Figure 5.10A) and a 7-fold increase ( $P < 0.001$ ) with bovine Hsp70 (Figure 5.10B) compared to control and which was followed by a subsequent decline in binding. An almost 2-fold decrease ( $P < 0.001$ ) was observed with bovine Hsp70 (Figure 5.10B), however despite the fold decrease a non-significant decline was observed with human Hsp72 (Figure 5.10A). In comparison, internalised HSPs significantly increased over time ( $P < 0.001$ , Figure 5.10A-B), until eighty minutes where a peak of internalisation, an almost 60-fold increase ( $P < 0.001$ ) with human Hsp72 (Figure 5.10A) and an 18-fold increase ( $P < 0.001$ ) with bovine Hsp70 (Figure 5.10B) was observed compared to the 0  $\mu\text{g/mL}$  control (Figure 5.10A-B). This was followed by a significant, 1-fold decline of internal bovine Hsp70 ( $P < 0.001$ , Figure 5.10B), however a non-significant decline of human Hsp72 (Figure 5.10A). A significant amount of human Hsp72 was found to bind to the cell surface of human erythrocytes at times  $> 10$  minutes ( $P < 0.001$ ) compared to bovine Hsp70 (Figure 5.10A-B) and to internalise more readily than bovine Hsp70 at times also  $> 10$  minutes ( $P < 0.05$ , twenty minutes, and  $P < 0.001$ ,  $> 20$  minutes, Figure 5.10A-B). In addition a significant correlation was also seen between surface-bound HSPs and internalised HSPs over time,  $r^2 = 0.8494$  ( $P < 0.001$ ) for human Hsp72 and  $r^2 = 0.4976$  ( $P < 0.05$ ) for bovine Hsp70 (Figure 5.10A-B).

The association binding rate of extracellular HSPs (0-50  $\mu\text{g/mL}$ ) to the erythrocyte cell surface (on non permeabilized cells) was also determined following incubation of erythrocytes with human Hsp72 (Figure 5.11) or bovine Hsp70 (Figure 5.12) for thirty minutes at 37°C. Following the incubation of human erythrocytes with extracellular HSPs, a significant increase in surface binding was seen at 1  $\mu\text{g/mL}$  human Hsp72 ( $P < 0.01$ , Figure 5.11) and bovine Hsp70 ( $P < 0.05$ , Figure 5.12) compared to control. The rate of binding was found to be dose-dependent at 1, 5, and 10  $\mu\text{g/mL}$  human Hsp72 and bovine Hsp72 when compared to control. Beyond these concentrations a plateau in expression was observed, where specific binding equalled specific binding at equilibrium for a certain concentration of HSP, between 25-50  $\mu\text{g/mL}$  HSP. An analysis of the kinetics of this uptake showed a typical rectangular hyperbola for surface binding

of human Hsp72 ( $R^2 = 0.9484$ , Figure 5.11) and bovine Hsp70 ( $R^2 = 0.9363$ , Figure 5.12), giving a KD (an affinity constant) of 7.080  $\mu\text{g/mL}$  Hsp72 and 10.15  $\mu\text{g/mL}$  Hsp70, suggesting human Hsp72 binds more avidly to erythrocytes than bovine Hsp70. Thus, similar to the previous experiments, these results suggest a significant amount of human Hsp72 binds to the cell surface of human erythrocytes ( $P < 0.001$ ) at concentrations  $> 0 \mu\text{g/mL}$  compared to bovine Hsp70 (Figures 5.11-5.12).

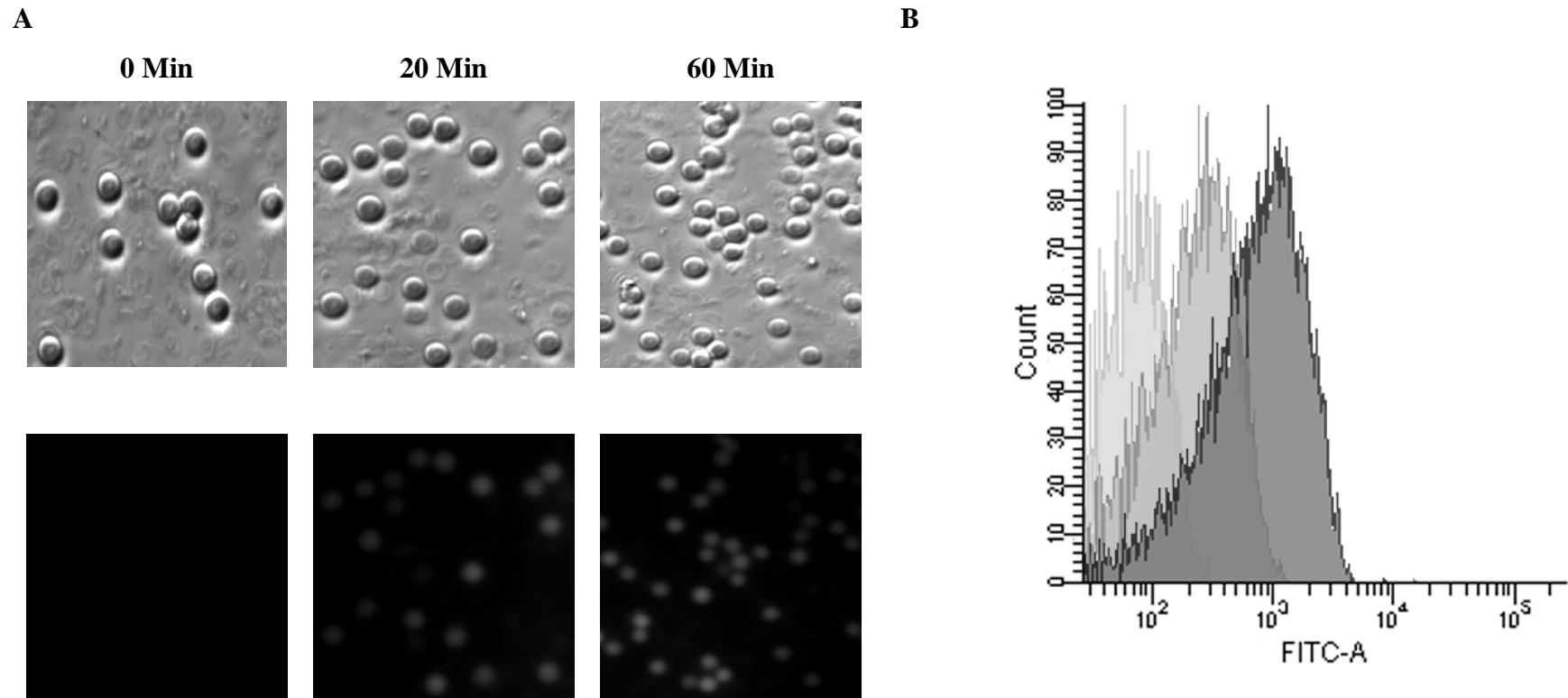
A reducing SDS-PAGE gel and Western blot of membrane bound Hsp72 and internalised Hsp72 (Figure 5.13) was performed. Clear bands appeared at approximately 70 kDa in samples containing isolated erythrocytes incubated for twenty or sixty minutes with 10  $\mu\text{g/mL}$  human Hsp72 at 37°C (Lanes 4 and 5, Figure 5.13A) indicating the presence of Hsp72 on erythrocytes. The SDS-PAGE gel shown in Figure 5.13A contains samples in which large smears and band distortion can be clearly seen (Lanes 3, 4 and 5). These samples contain the erythrocyte membrane extract, which contain the protein of interest. The Western blot of erythrocyte membranes probed with a specific antibody for Hsp72 (Figure 5.13B) showed clear bands at approximately 70 kDa in the samples containing an erythrocyte membrane extract incubated with human Hsp72 for twenty minutes at 37°C, and a more intense band in the sample containing an erythrocyte membrane extract incubated with Hsp72 for sixty minutes at 37°C (Lanes 4 and 5). Untreated erythrocyte membrane extract at 37°C showed no band intensity (Lane 3) and all intracellular erythrocyte extracts again showed no band intensity (Lanes 8, 9 and 10).

**Table 5.1: The binding of extracellular HSPs to non-permeabilized or permeabilized human erythrocytes.**

Human erythrocytes were incubated with 10 µg/mL human Hsp72 or bovine Hsp70 for 1 hour at 37°C. Surface binding (on non permeabilized cells) and internalisation (on/inside permeabilized cells) of HSPs was then determined by flow cytometry.

Treatment	HSP Mean Fluorescence Intensity (MFI)	
	Surface	Internal
<b>0 µg/mL HSP</b>	16 ± 2	18 ± 3
<b>10 µg/mL Hsp72</b>	787 ± 11 ***	1077 ± 8 ***
<b>10 µg/mL Hsp70</b>	542 ± 5 ***	836 ± 2 ***

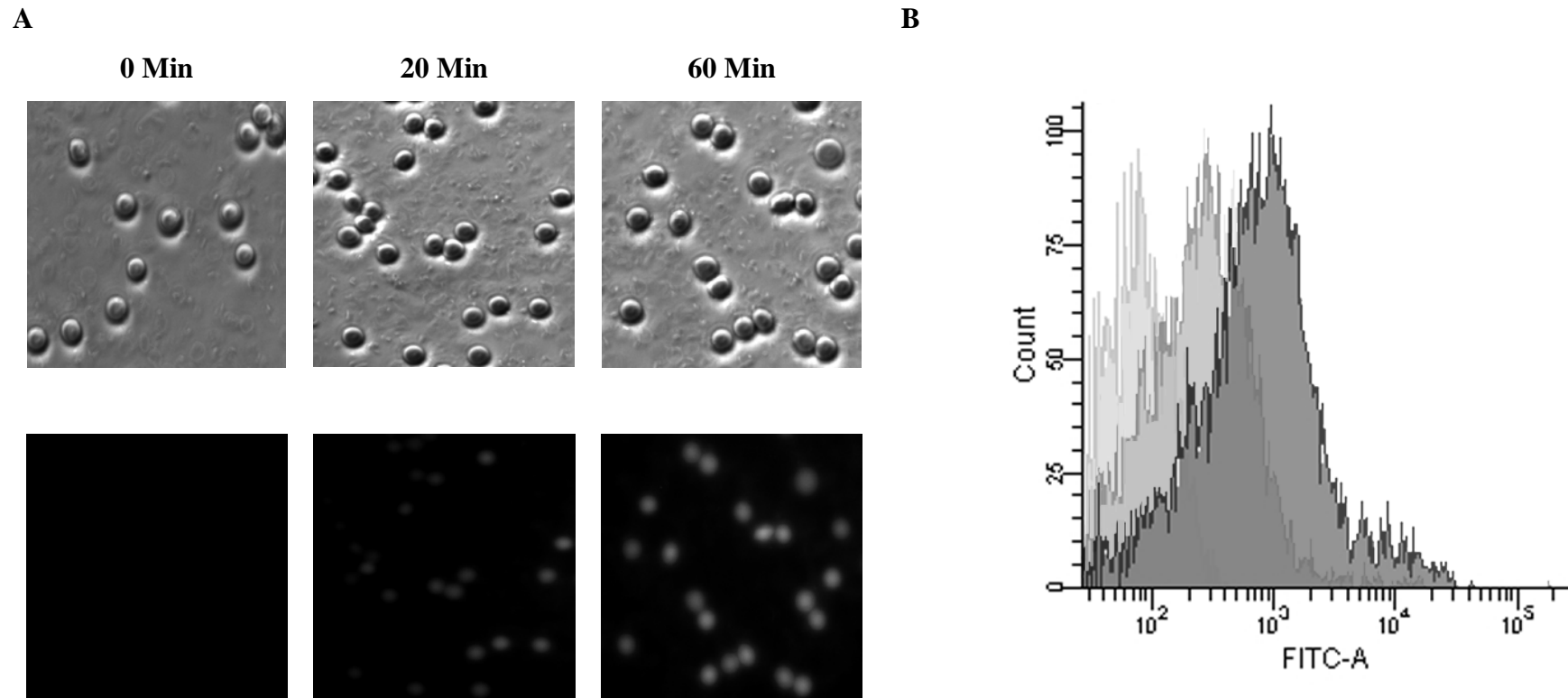
Note: Data are presented as SEM, n=3. Means of surface and internal human Hsp72 and bovine Hsp70 were separately compared to 0 µg/mL controls through use of one-way ANOVA with Bonferroni's *post hoc* test, \*\*\* (P<0.001).



**Figure 5.8: The binding of extracellular Hsp72 to non-permeabilized human erythrocytes in a time dependent manner.**

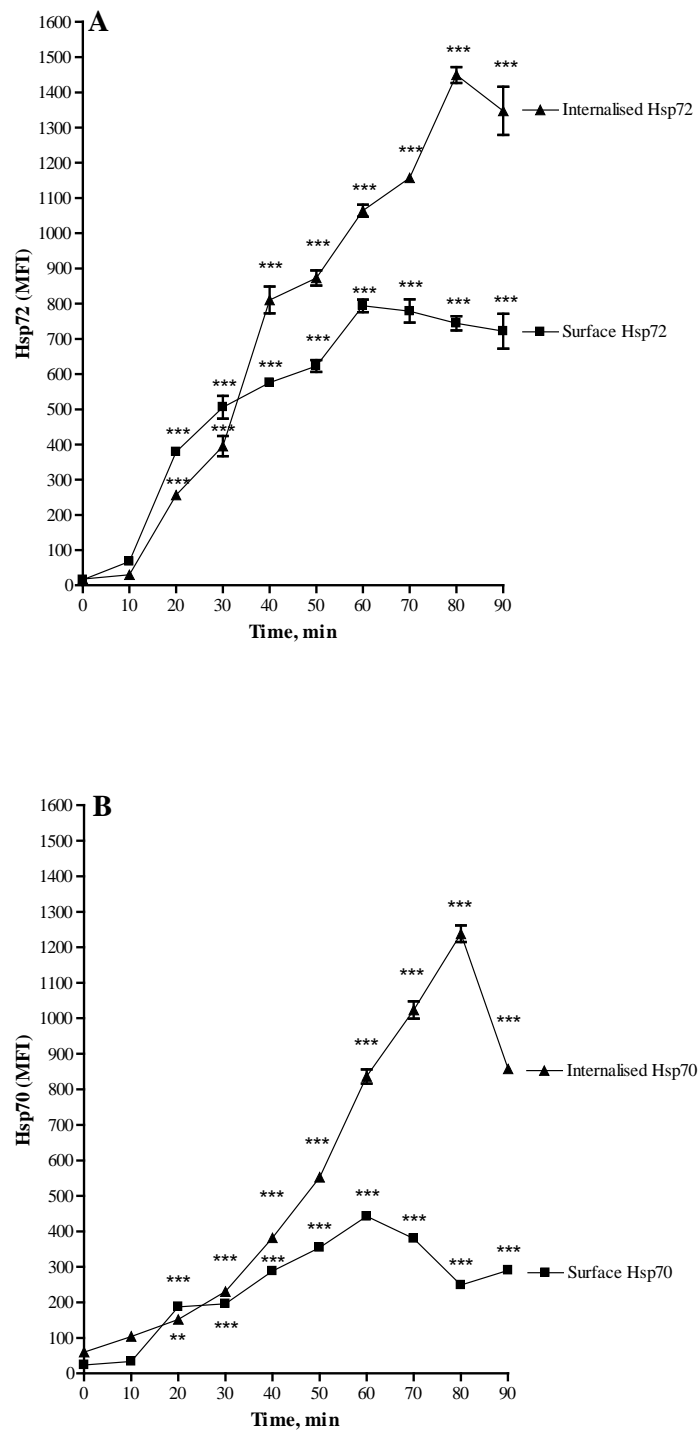
Human erythrocytes were incubated with recombinant human Hsp72 (10  $\mu\text{g/mL}$ ) for 0, 20 or 60 minutes at 37°C and surface binding of Hsp72 (**A**) was determined by fluorescence microscopy and (**B**) flow cytometry. Histograms represent: control (■), cells incubated with human Hsp72 for 20 minutes (■), and cells incubated with human Hsp72 for 60 minutes (■). Images are representative of six independent experiments.





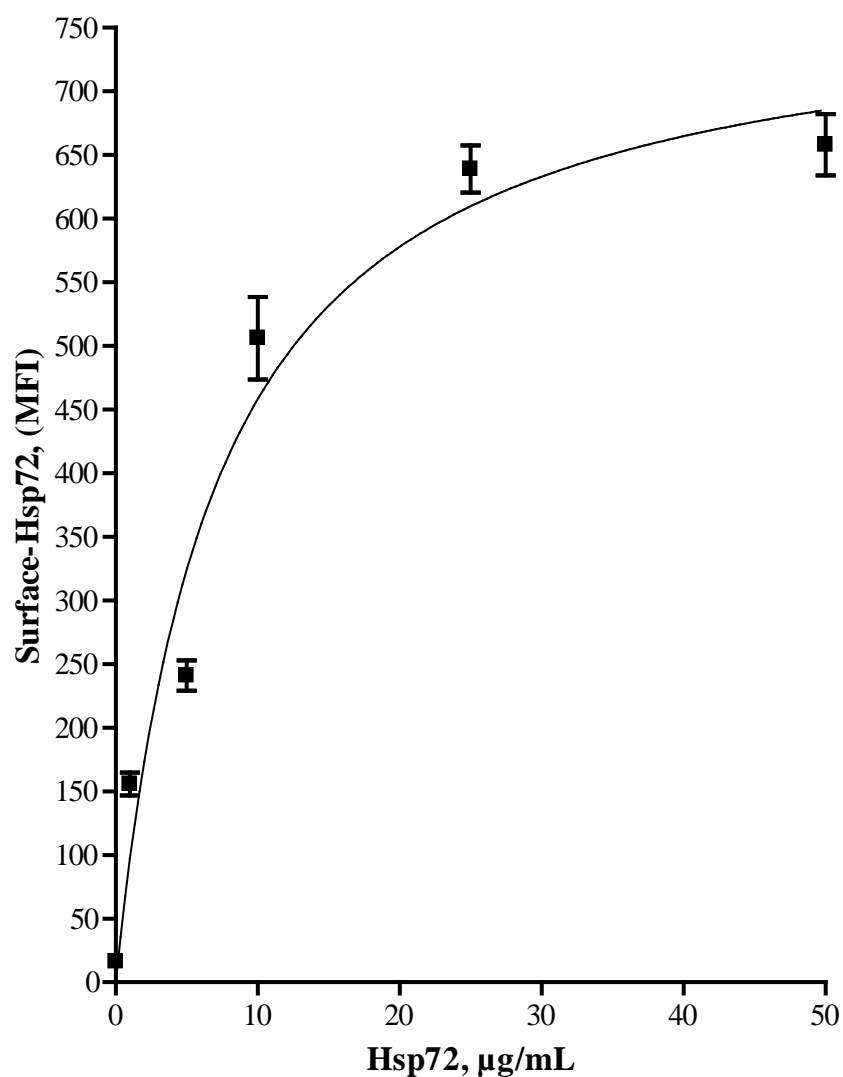
**Figure 5.9: The binding of extracellular Hsp72 to permeabilized human erythrocytes in a time dependent manner.**

Human erythrocytes were incubated with recombinant human Hsp72 (10  $\mu\text{g/mL}$ ) for 0, 20 or 60 minutes at 37°C and surface binding of Hsp72 to permeabilized cells (**A**) was determined by fluorescence microscopy and (**B**) flow cytometry. Histograms represent: control (■), cells incubated with human Hsp72 for 20 minutes (■), and cells incubated with human Hsp72 for 60 minutes (■). Images are representative of six independent experiments.



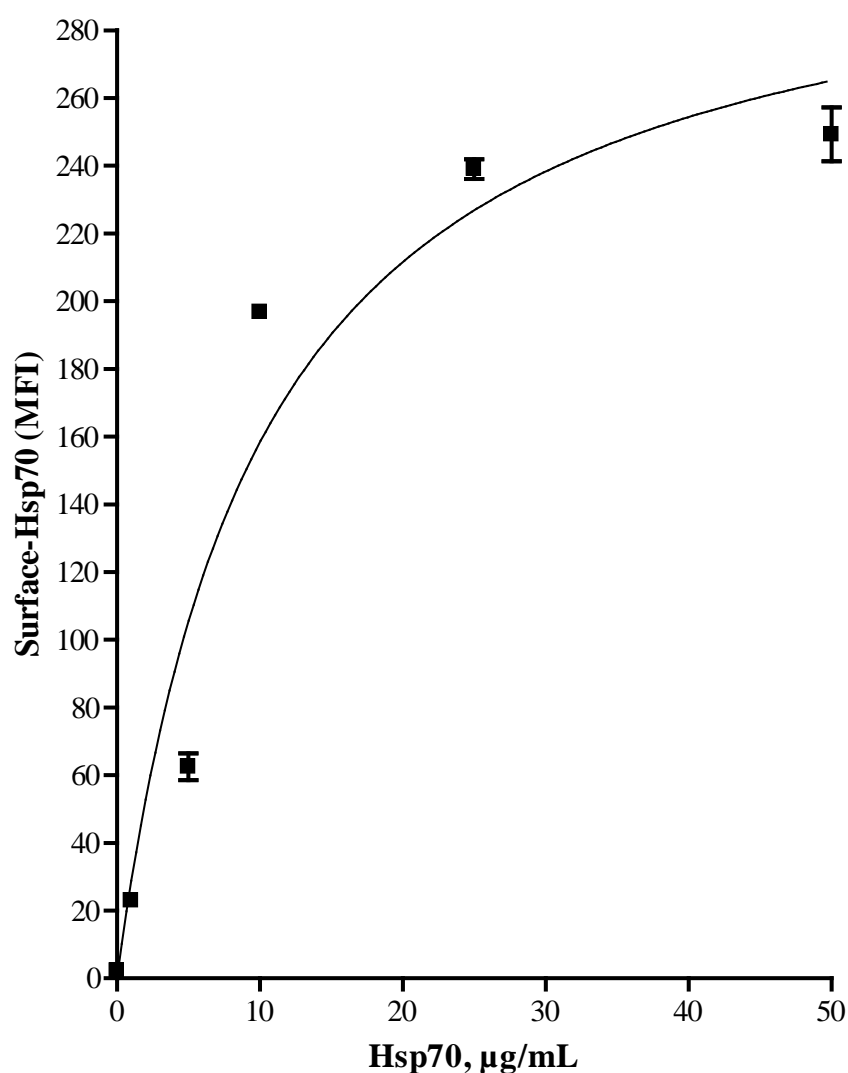
**Figure 5.10: Surface binding and internalisation of HSPs in human erythrocytes in response to 0-90 minute incubation with 10  $\mu\text{g/mL}$  human Hsp72 (A) and bovine Hsp70 (B) at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean time points compared to time zero through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ). Pearson correlation,  $r^2 = 0.9216$  (Human Hsp72),  $r^2 = 0.4976$  (Bovine Hsp70).



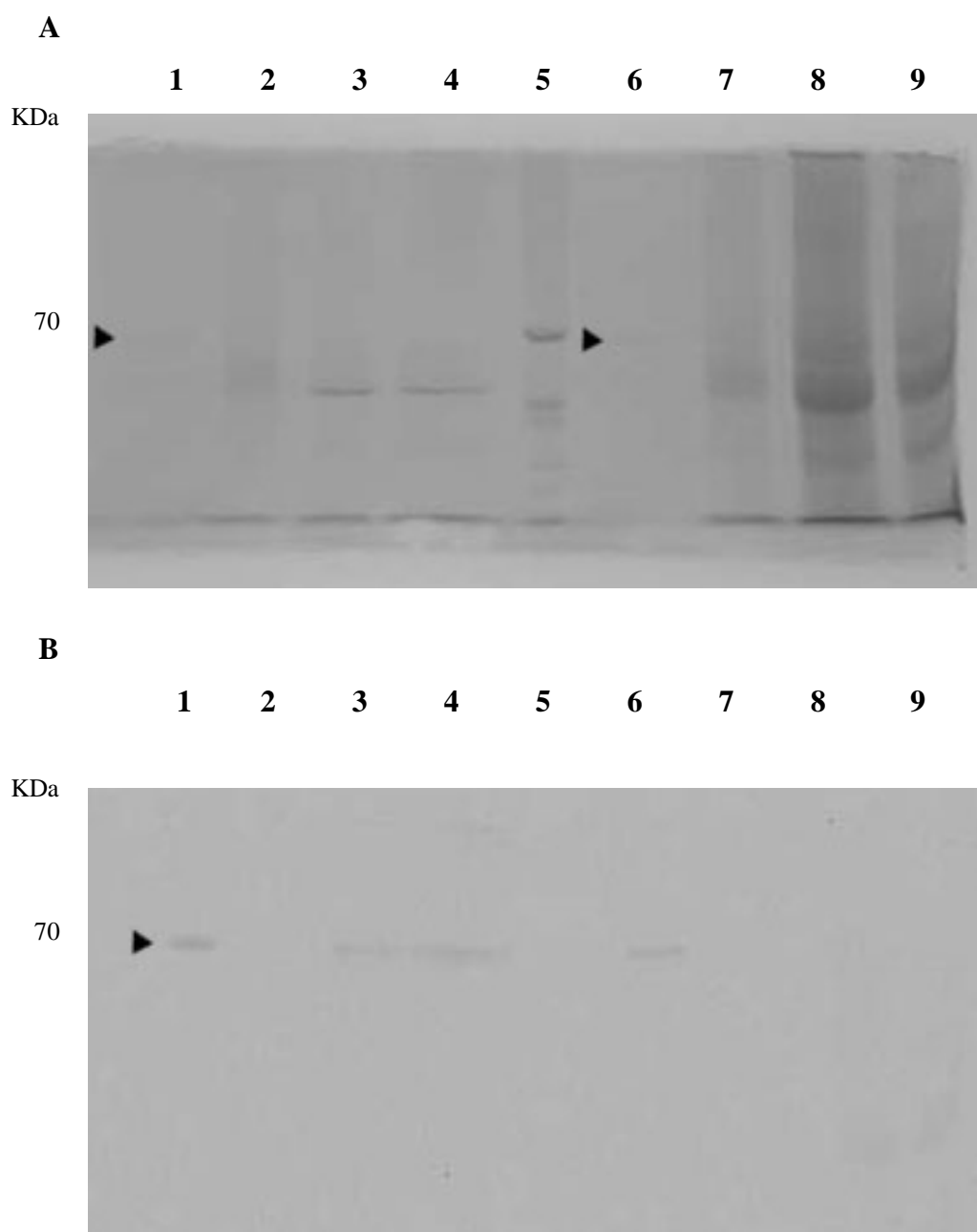
**Figure 5.11: Surface binding of Hsp72 to human erythrocytes following 30 minute incubation with 0-50  $\mu\text{g/mL}$  human Hsp72 at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Data was fitted in a rectangular hyperbola assuming one site binding. A.VMAX: 782.3,  $\pm$  SEM: 47.32, KD: 7.080  $\pm$  SEM: 1.429 and  $R^2$ : 0.9484.



**Figure 5.12: Surface binding of Hsp70 in human erythrocytes following 30 minute incubation with 0-50  $\mu\text{g/mL}$  bovine Hsp70 at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=4$ . Data was fitted in a rectangular hyperbola assuming one site binding. A.VMAX: 318.19,  $\pm$  SEM: 22.94, KD: 10.15  $\pm$  SEM: 2.149 and  $R^2$ : 0.9363.



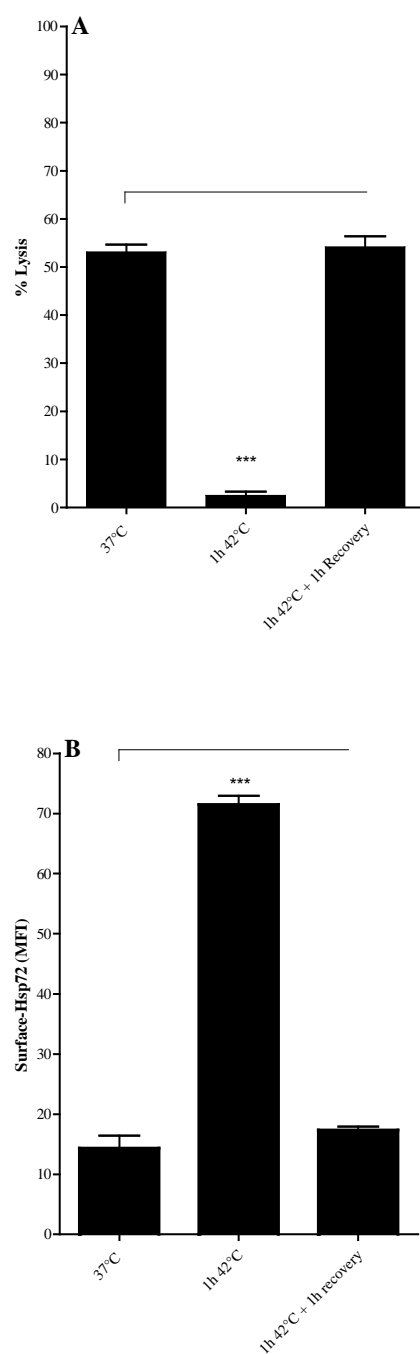
**Figure 5.13: Reducing SDS-Page gel (A) and Western blot (B) of membrane associated and internalised Hsp72 protein from human erythrocytes.**

A 10 % gel was cast and loaded with the following under reduced conditions: Lane 1 - pure human Hsp72 (1 µg/mL); Lane 2 - erythrocyte membrane extract at 37°C; Lane 3 - erythrocyte membrane extract incubated with Hsp72 for 20 minutes at 37°C (1 µg/mL); Lane 4 - erythrocyte membrane extract incubated with Hsp72 for 60 minutes at 37°C (1 µg/mL); Lane 5 - molecular weight standards; Lane 6 - pure human Hsp72 (1 µg/mL); Lane 7 - cell lysate at 37°C; Lane 8 - cell lysate incubated with Hsp72 for 20 minutes at 37°C (1 µg/mL) and Lane 9 - cell lysate incubated with Hsp72 for 60 minutes at 37°C (1 µg/mL). The gel was stained using Imperial Protein Stain. The blot was probed with 1:2000 dilution of biotinylated anti-Hsp72 followed by avidin at 1:5000 dilution and was stained with SuperSignal® west pico chemiluminescent substrate and exposed for 480 seconds.

#### **5.3.4 Erythrocyte fragility and Hsp72 detection following one hour *in vivo* heat treatments.**

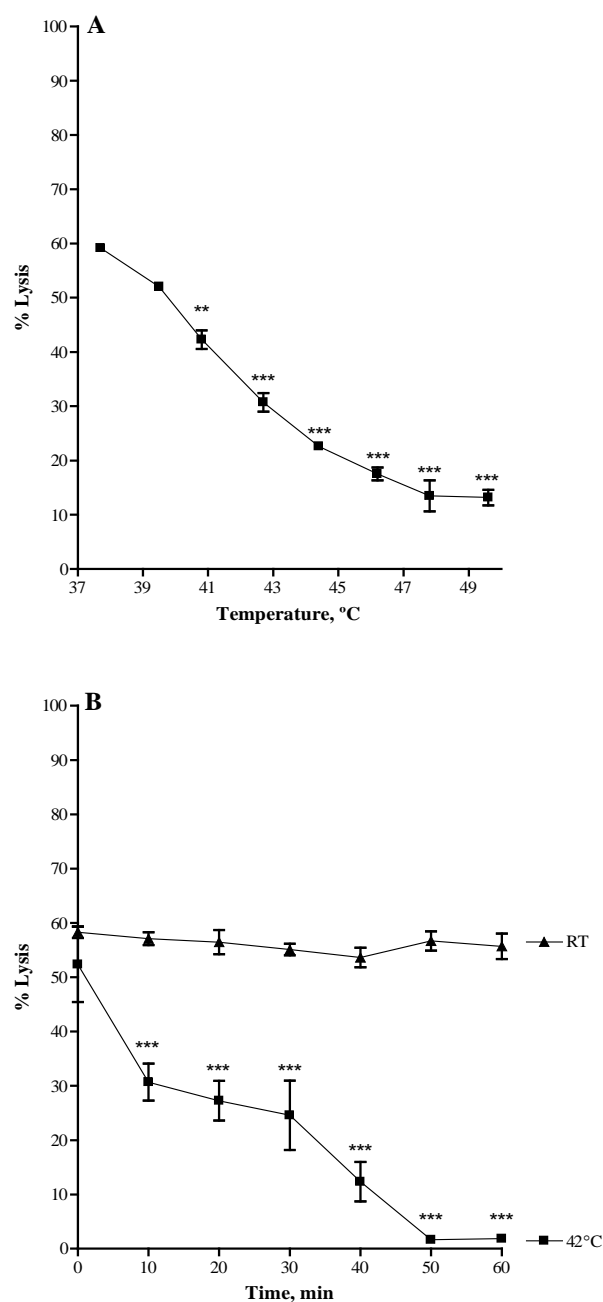
Erythrocyte fragility and surface binding of Hsp72 on non-permeabilized cells following treatments with heat shock are presented in Figures 5.14-5.16.

Following the results of the *in vitro* experiments it was then investigated whether human erythrocytes can be protected by Hsp72 *in vivo* by inducing an increase in local body temperature through the immersion of healthy volunteers' hands in a hot water bath for one hour at 42°C. Peripheral blood was taken prior to heat exposure, immediately following the heat shock treatment, and after a one hour recovery period at 37°C. The one hour heat shock treatment at 42°C resulted in a significant reduction in membrane fragility of human erythrocytes ( $P < 0.001$ , Figure 5.14A), which returned to normal values after one hour recovery at 37°C (Figure 5.14A). After treatment at 42°C, a significant increase in the presence of surface bound Hsp72 on non-permeabilized cells ( $P < 0.001$ , Figure 5.14B) was observed, which again decreased after one hour recovery at 37°C to values of that of control cells (Figure 5.14B). The *in vivo* experiment was then adapted using individual fingers at different temperatures, in a temperature gradient bar for one hour, after which blood was taken and erythrocyte fragility measured. A significant degree of protection was seen at temperatures greater than 39.5°C ( $P < 0.01$ , Figure 5.15A), and this increased with temperature ( $P < 0.001$ , Figure 5.15A), although greater than 50 % protection was not achieved below 42.7°C (Figure 5.15A). Above 42.7°C there was a sharp increase in protection to over 90 % protection at 47.8°C ( $P < 0.001$ , Figure 15A). A time course using whole hand immersion at 42°C for one hour in a hot water bath, demonstrated that significant protection from osmotic shock occurred within ten minutes ( $P < 0.001$ , Figure 5.15B), and that complete protection was achieved at fifty minutes ( $P < 0.001$ , Figure 5.15B). The erythrocytes taken from these volunteers' hands maintained at room temperature were not protected from osmotic shock (0 % protection). There was no change in the red blood cell counts during any of these experiments.



**Figure 5.14: Cell lysis (A) and Hsp72 binding (B) on human erythrocytes, following 1 hour heat shock treatment (42°C).**

Finger prick blood was taken prior to immersion, following 1 hour treatment (42°C) and 1 hour recovery at 37°C. Erythrocyte fragility was determined by absorbance 550 nm following addition of 3.5 mM NaCl (A). Surface binding of Hsp72 on non-permeabilized cells was determined by flow cytometry (B). Data are presented as mean  $\pm$  SEM,  $n=4$ . Significance shown as difference from 37°C control using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



**Figure 5.15: Hsp72 protection of human erythrocytes *in vivo* following localised heat shock treatment.**

(A) The individual fingers of volunteers were subjected to 1 hour heat shock 37.7°C-49.6°C in a thermal gradient bar (allowing each finger to be at a different temperature), n=2. (B) Volunteers placed one hand into a water bath at 42°C for 1 hour. Peripheral blood was taken at 10 minute intervals following immersion from the heat treated and hand maintained at RT, n=3. Data are presented as mean  $\pm$  SEM. \* Indicates significant difference between mean temperature or time points, compared to 37.7°C or 0 minute controls, through the use of one-way or two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* (P<0.01), \*\*\* (P<0.001).



### 5.3.5 Potential receptors for extracellular HSPs on human erythrocytes.

Detection of Hsp72 and Hsp70 surface receptors on non-permeabilized erythrocytes are presented in Figures 5.16-5.19 and Table 5.2.

The following experiments identified potential membrane receptors on human erythrocytes to which Hsp72 binds by a series of *in vitro* competition experiments. Erythrocytes were exposed to human Hsp72 (0 or 10 µg/mL) for one hour at 37°C alone or in combination with a pre-incubation of Annexin V for twenty minutes, or anti-CD77, BSA or malBSA for one hour at 37°C. Erythrocytes incubated with human Hsp72 (10 µg/mL) for one hour at 37°C were protected from osmotic shock and surface bound Hsp72 (on non-permeabilized cells) was easily detected (Table 5.2). In comparison, erythrocytes exposed to Annexin V, anti-CD77, BSA or malBSA alone were not protected from osmotic shock and surface bound Hsp72 was not detected (Table 5.2). Furthermore, the lipids PS (detected using Annexin V) and globotriaosylceramide (Gb3 or CD77) (detected using anti-CD77), which have been previously identified as binding partners for Hsp72 (Arispe et al. 2004; Gehrmann et al. 2008), were not detectable on the erythrocyte membrane (Table 5.2). Competition experiments revealed pre-incubation of cells for twenty minutes with Annexin V, or anti-CD77 or BSA for one hour prior to Hsp72 treatment, did not prevent Hsp72 mediated protection from osmotic shock or binding of human Hsp72 to the erythrocyte membrane, suggesting that Hsp72 is not interacting with either PS or Gb3 in this system (Table 5.2). However, malBSA significantly prevented the Hsp72 mediated decrease in membrane fragility ( $P<0.01$ , Table 5.2), and also significantly inhibited the binding of human Hsp72 to the erythrocyte membrane ( $P<0.01$ , Table 5.2). As suggested in chapter four, malBSA binds with high affinity to several SRs. These results therefore provided initial evidence for the existence of SRs on human erythrocytes. To identify which SRs might be involved antibodies against the SRs: LOX-1 and SR-A1, and two SR ligands, fucoidan and poly (I) were utilised. Competitive inhibition experiments demonstrated no effect on erythrocyte fragility or human Hsp72 surface binding to the erythrocyte membrane with anti-LOX-1 (Table 5.2). Furthermore, erythrocytes exposed to anti-LOX-1 alone were not protected from osmotic shock, surface bound human Hsp72 was not detected

(Table 5.2), and the presence of LOX-1 on the erythrocyte membrane was also not detectable (Table 5.2). However, anti-SR-A1, fucoidan and poly (I) were all able to significantly block Hsp72 binding, and prevented Hsp72 mediated decrease in membrane fragility ( $P<0.01$ , Table 5.2).

Fucoidan and poly (I) bind to a range of SRs, but have a higher affinity for SR-A receptors. There are no previous reports of SR-A receptors on erythrocytes, so the specific anti-SR-A1 antibody with a secondary FITC conjugated IgG antibody were used for indirect detection. The significant increase in MFI observed ( $P<0.001$ , Figures 5.16A-5.17B and Table 5.2) compared to no stain control cells confirmed the presence of SR-A1 receptors on erythrocyte membranes using flow cytometry, confirmed by fluorescence microscopy (Figure 5.16B). Direct competitive inhibition experiments between human Hsp72 and anti-SR-A1 were also performed using flow cytometry (Figure 5.17A-B) and fluorescence microscopy (Figure 5.18). Using flow cytometry, competition for the SR-A1 receptor on erythrocyte membranes resulted with a significant decrease in human Hsp72 surface binding compared to control cells ( $P<0.01$ , Figure 5.17A), when erythrocytes were pre-incubated for one hour with 10  $\mu\text{g/mL}$  anti-SR-A1 at 37°C (Figure 5.17A). In comparison, when erythrocytes were pre-incubated for one hour with human Hsp72 followed by incubation with anti-SR-A1 at 37°C there was no effect on human Hsp72 surface binding (Figure 5.17A). The SR-A1 signal on erythrocyte membranes was also successfully masked by prior addition of 10  $\mu\text{g/mL}$  human Hsp72 for one hour to human erythrocytes at 37°C ( $P<0.001$ , Figure 5.17B) compared to control cells. In contrast, erythrocytes pre-incubated for one hour with anti-SR-A1 followed by incubation with human Hsp72 at 37°C had no effect on the SR-A1 signal (Figure 5.17B), further supporting the role of the SR-A1 receptor in the Hsp72 mediated protection. Surface binding of anti-SR-A1 to the SR-A1 receptor was also investigated using fluorescence microscopy (Figure 5.18). These experiments identified erythrocytes incubated for one hour with 10  $\mu\text{g/mL}$  bovine Hsp70 followed by incubation with 10  $\mu\text{g/mL}$  anti-SR-A1, resulted in reduced binding of anti-SR-A1 to the SR-A1 receptor which continued to decrease with increasing bovine Hsp70 concentration, 50 and 100  $\mu\text{g/mL}$  (Figure 5.18), again

supporting the role of the SR-A1 receptors in the Hsp72 surface binding and function.

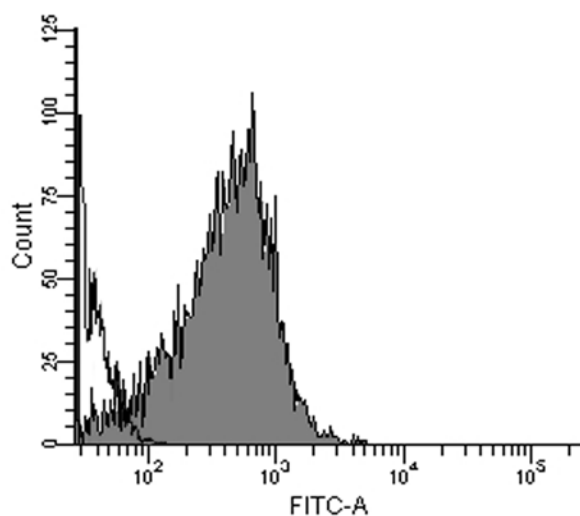
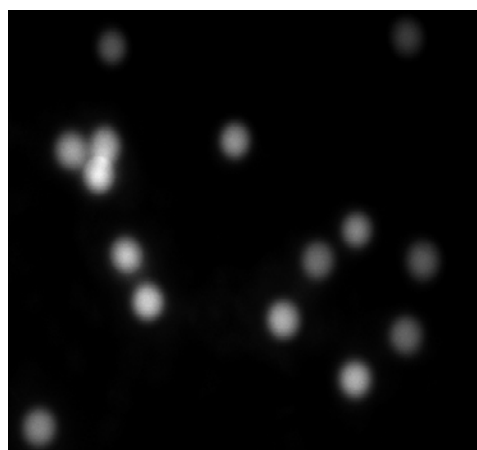
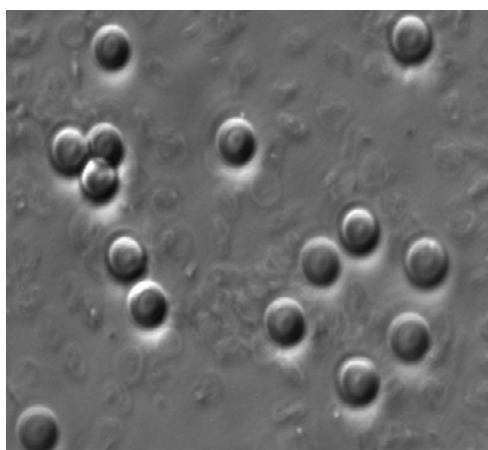
A reducing SDS-PAGE gel and Western blot of the membrane receptor SR-A1 (Figure 5.19) was performed to confirm the presence of the receptor on erythrocyte membranes. Bands should normally be seen at approximately 250, 150 or 77 kDa depending on the degree of denaturation (Collot-Teixeira et al. 2007). The SDS-PAGE gel shows two clear band at approximately 150 and 77 kDa in samples containing U937 macrophage membrane extracts - used as a control and erythrocyte membrane extracts (Lanes 1 and 3, Figure 5.19A) indicating intact SR-A1 at 37°C. Similar to previous SDS-PAGE gels, bands appear distorted due to the membrane extraction process. The Western blot of erythrocyte membranes probed with a specific antibody for SR-A1 (Figure 5.19B) shows clear bands at approximately 150 and 77 kDa in the samples containing U937 macrophage membrane extracts and erythrocyte membrane extracts at 37°C (Lane 1 and 3). The intracellular samples of U937 macrophages and erythrocyte extracts showed decreased band intensity (Lanes 2 and 4).

**Table 5.2: SR-A1 is a target for Hsp72 binding on human erythrocytes.**

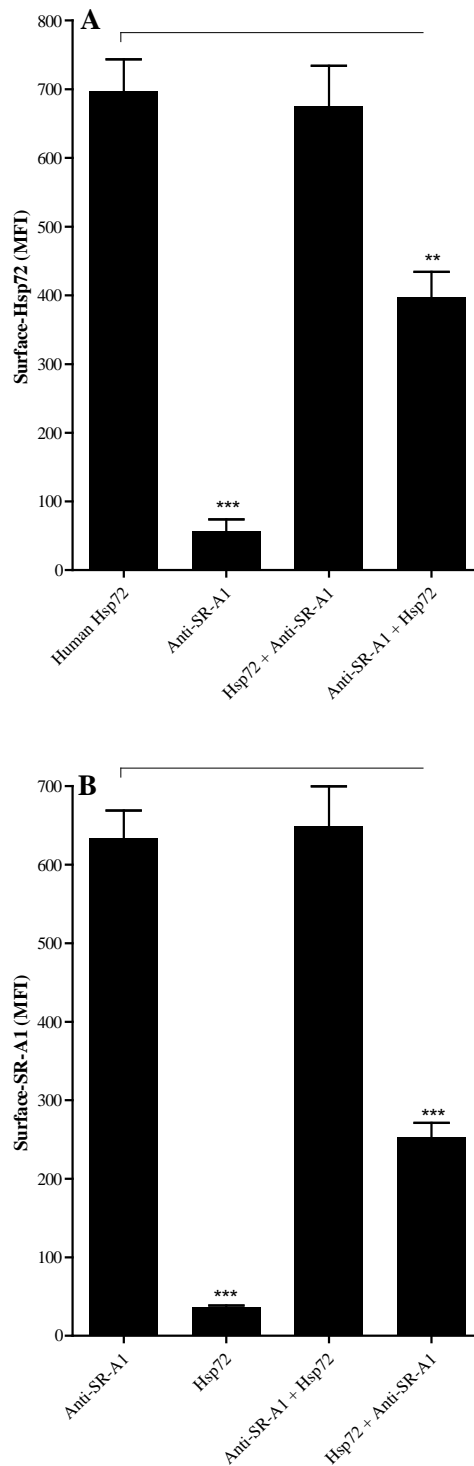
Erythrocytes were incubated with 10 µg/mL human Hsp72 at 37°C for 1 hour, following pre-incubation with antibodies or ligands to potential Hsp72 receptors for either 20 minutes or 1 hour at 37°C. Cell lysis was determined by absorbance at 550 nm and surface binding (on non-permeabilized cells) of human Hsp72 was determined by flow cytometry.

<b>Treatment</b>	<b>Osmotic fragility (Percentage lysis)</b>	<b>Bound Hsp72 (MFI)</b>	<b>Antibody detection (MFI)</b>
<b>Control</b>	45.8 ± 0.2 **	ND	-
<b>Human Hsp72</b>	2.3 ± 0.2	695 ± 17	-
<b>Annexin V</b>	42.8 ± 0.2 **	ND	2 ± 1
<b>Anti-CD77</b>	44.0 ± 5.8 **	ND	3 ± 1
<b>BSA</b>	41.3 ± 0.3 **	ND	-
<b>malBSA</b>	36.0 ± 1.2 **	ND	-
<b>Anti-LOX-1</b>	38.7 ± 1.4 **	ND	12 ± 1
<b>Fucoidan</b>	38.8 ± 0.9 **	ND	-
<b>Poly (I)</b>	40.5 ± 1.8 **	ND	-
<b>Anti-SR-A1</b>	48.2 ± 0.5 **	ND	418 ± 8
<b>Annexin V, Hsp72</b>	2.6 ± 0.3	686 ± 17	-
<b>Anti-CD77, Hsp72</b>	4.5 ± 0.5	691 ± 13	-
<b>BSA, Hsp72</b>	2.6 ± 0.4	683 ± 27	-
<b>malBSA, Hsp72</b>	34.0 ± 0.3 **	227 ± 14 **	-
<b>Anti-LOX-1, Hsp72</b>	2.8 ± 0.2	647 ± 4	-
<b>Fucoidan, Hsp72</b>	35.3 ± 0.6 **	381 ± 6 **	-
<b>Poly (I), Hsp72</b>	32.3 ± 0.3 **	343 ± 2 **	-
<b>Anti-SR-A1, Hsp72</b>	46.6 ± 1.0 **	318 ± 2 **	-

Note: Data are presented as mean ± SEM, n=3. Means were compared for each variable against the human Hsp72 control through use of one-way ANOVA with Dunnett's *post hoc* test, \*\* (P<0.01). ND indicates not detectable.

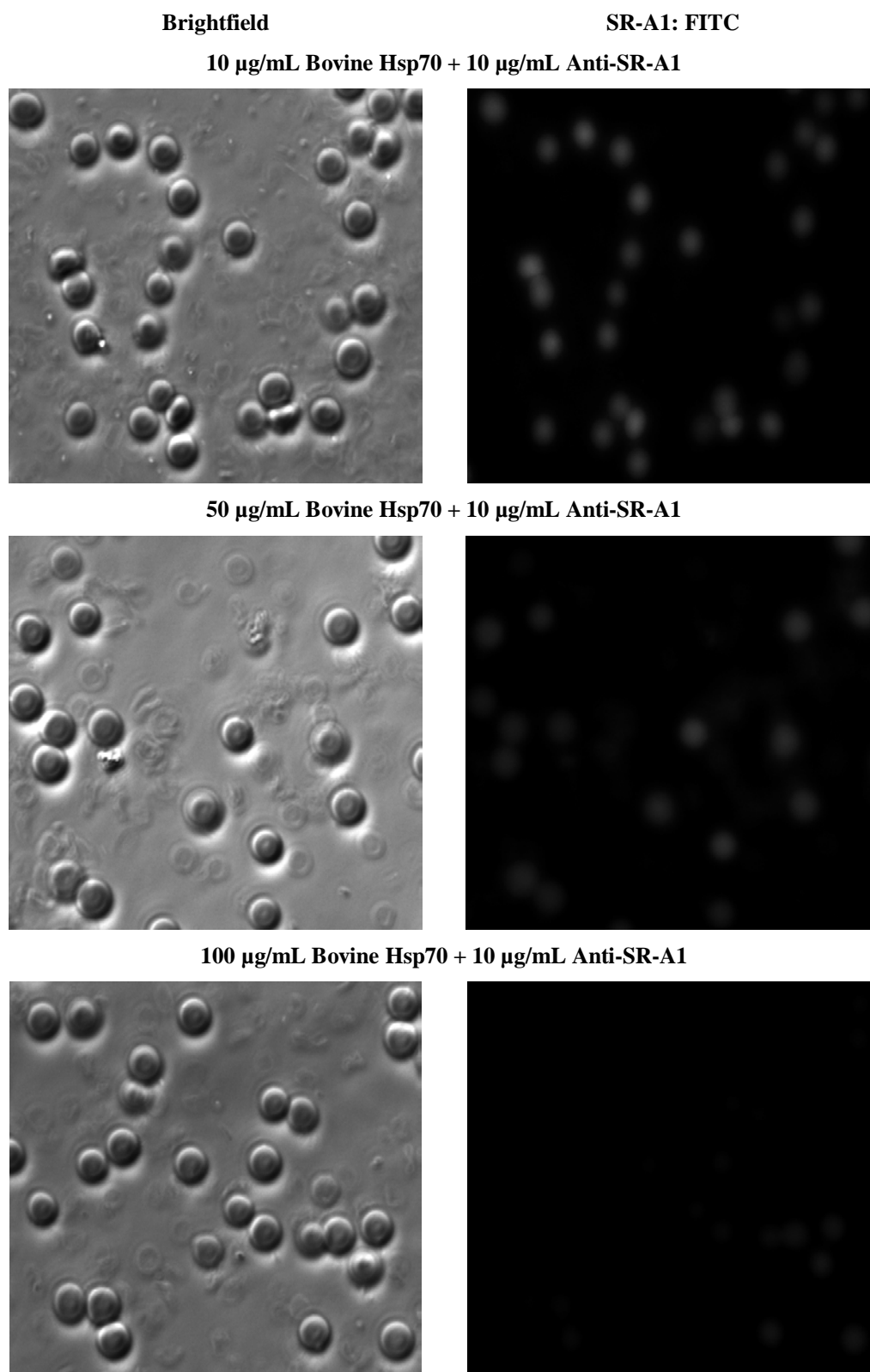
**A.****B.****Brightfield****SR-A1: FITC****10  $\mu$ g/mL Anti-SR-A1****Figure 5.16: SR-A1 is present in human erythrocyte membranes.**

Human erythrocytes were incubated with 10  $\mu$ g/mL anti-SR-A1 for 1 hour at 37°C, followed by the addition of an IgG FITC conjugated antibody for 1 hour at 37°C. Surface binding was determined by flow cytometry (**A**) and fluorescence microscopy (**B**). Histograms represent: control ( $\square$ ) or cells stained with anti-SR-A1 antibody ( $\blacksquare$ ). Fluorescence microscopy images were acquired at a magnification of x 60 set up in FITC (SR-A1 stain)-fluorescence mode maintained at 25°C. Histograms and images are representative of three independent experiments.



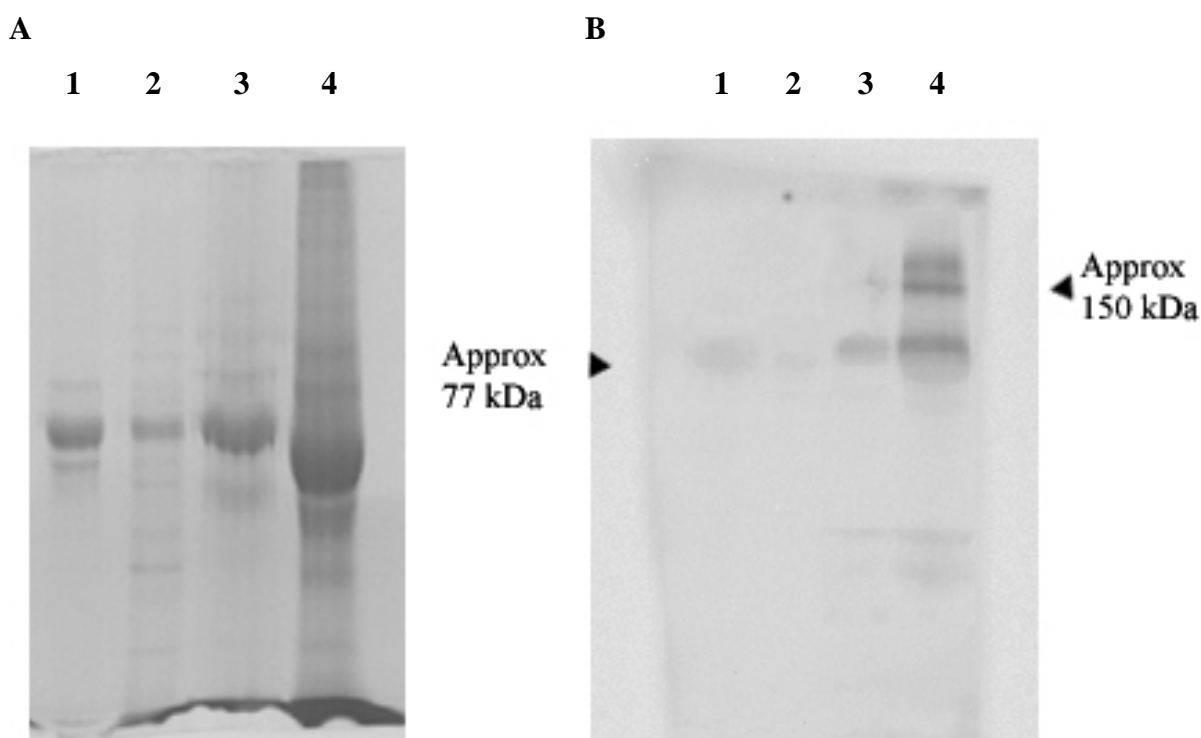
**Figure 5.17: Surface binding of human Hsp72 (10  $\mu\text{g/mL}$ ) on human erythrocytes before or after the addition of anti-SR-A1 (10  $\mu\text{g/mL}$ ) at 37°C (A), or surface binding of anti-SR-A1 (10  $\mu\text{g/mL}$ ) on human erythrocytes before or after the addition of human Hsp72 (10  $\mu\text{g/mL}$ ) at 37°C (B).**

Data are presented as mean  $\pm$  SEM,  $n=3$ . \* Indicates significant difference compared to human Hsp72 (A) or anti-SR-A1 (B) control by one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



**Figure 5.18: Competition assay between bovine Hsp70 and anti-SR-A1 at 37°C.**

Human erythrocytes were incubated for 1 hour with 10-100  $\mu\text{g/mL}$  bovine Hsp70 at 37°C followed by 1 hour incubation with 10  $\mu\text{g/mL}$  anti-SR-A1 and IgG FITC conjugated antibody at 37°C. Anti-SR-A1 binding on human erythrocytes was determined by fluorescence microscopy, acquired at a magnification of x 60 set up in FITC (SR-A1 stain)-fluorescence mode maintained at 25°C. Data are representative of three independent experiments.



**Figure 5.19: Reducing SDS-Page gel (A) and Western blot (B) of membrane SR-A1 protein from human erythrocytes.**

A 10 % gel was cast and loaded with the following under reduced conditions: Lane 1 - U937 macrophage membrane extract at 37°C; Lane 2 - U937 macrophage lysate extract at 37°C; Lane 3 - erythrocyte membrane extract at 37°C; Lane 4 - erythrocyte lysate extract at 37°C. The gel was stained using Imperial Protein Stain. The blot was probed with 1:2000 dilution of anti-SR-A1 followed by anti-goat IgG peroxidase at 1:5000 dilution, and stained with SuperSignal® west femto chemiluminescent substrate and blots were exposed for 480 seconds.



## 5.4 Discussion

The aims of this chapter were to determine whether extracellular bovine Hsp70 or human Hsp72 were able to protect human erythrocytes from osmotic stress, uric acid or elevated temperature stress *in vitro* followed *in vivo* and *ex vivo* experiments. Also, the possible cell surface interactions by which the HSPs could afford this protection was investigated.

### 5.4.1 Extracellular HSPs stabilise erythrocyte membranes.

Studies in the last two decades have revealed under normal conditions Hsp72 is present in human serum (Pockley et al. 1998; Wright et al. 2000; Hunter-Lavin et al. 2004b), the level of which is affected by some disease states including carotid atherosclerosis, peripheral and renal disease and coronary artery disease (Wick et al. 1995; Pockley et al. 2003b; Hunter-Lavin et al. 2004b; Wright et al. 2000; Zhu et al. 2003). Several reasons for this extracellular presence have been proposed and one potential role is the stabilisation of cell membranes, including erythrocytes. The data in this chapter demonstrates a protective effect of exogenous bovine Hsp70 and recombinant human Hsp72 *ex vivo* on erythrocytes induced to haemolysis with elevated temperatures equivalent to fever (Marinkovic et al. 2009), osmotic shock using hypotonic concentrations of NaCl (Bolton, 1949; Penha-Silva et al. 2007), and increased levels of the potent pro-oxidant uric acid (Rowlett, 2001), supporting the theory of Hsp70-mediated cell protection against factors inducing cell death (Guzhova et al. 1998). The results indicate that a number of different concentrations of bovine Hsp70 or human Hsp72 proteins (0-30 µg/mL) applied to isolated human erythrocytes over a period of four hours prior to physiological and environmental stresses which cause physical changes in the erythrocyte membrane leading haemolysis: temperature, osmotic and uric acid exposure (Baar, 1967; Tsong & Kingsley, 1975; Gershfeld & Murayama, 1988; Penha-Silva et al. 2007; Marinkovic et al. 2009), prevented erythrocyte lysis and decreased the fragility of the erythrocyte membrane. Therefore, Hsp72 was able to protect erythrocytes from three different stressors. These data suggest extracellular Hsp70 or Hsp72 to have a functional role in the protection of the erythrocyte cell membrane from *in vitro* stress through stabilising membrane physical structure. Studying the erythrocyte membrane was easily established since it could be lysed with ease, because only

one membrane is involved composed of three layers surrounded by a macroscopically homogenous cytoplasmic space, releasing the contents of the membrane (haemoglobin), due to osmotic lysis. This, according to Maclean, (1978) allows for one of the purest fractions of the cell membrane available to an experimenter. In this chapter, haemoglobin leakage was used for determining haemolysis and was an ideal measurement of membrane damage. In these experiments, a dose-dependent protection was induced, with 1 µg/mL human Hsp72 being the most physiologically relevant concentration to induce protection from heat stress and uric acid exposure, whilst <1 µg/mL was found to induce protection from osmotic stress. In no instance did the control protein, BSA, at any concentration give a comparable effect with human erythrocytes. This form of protection is very similar to that observed in other studies where externally supplied Hsp72 has been shown to increase cell resistance to apoptosis *in vitro* in a number of cultured cell lines. These include monocytes (Guzhova et al. 1998), neuroblastoma cells (Guzhova et al. 2001) and aortic smooth muscle cells (Johnson et al. 1990). When HSP70 proteins, whether bovine or human, were applied to erythrocytes, there was a high correlation between HSP concentration and incubation time ( $r^2 = 0.9471$ ), with human Hsp72 and/or bovine Hsp70 effects occurring within a one hour incubation, suggesting the mechanism by which HSP works is through interaction with the cell surface and/or internalisation. A previous study by Tytell and colleagues (1986) demonstrated the transfer of glia-axon transfer proteins from adjacent periaxonal glia cells into the squid giant axon. Some of these proteins were found to be Hsp72 and Hsc70 (Tytell et al. 1986). This finding and the possibility that glia can provide neurons with HSP based protection prompted the study by Guzhova et al. (2001). They found human neuroblastoma cells in the central nervous system, which are chronically depleted of Hsp70, to take up soluble Hsp72 *in vitro* in response to heat stress. The neuroblastoma cells were found to have decreased levels of Hsp70 because of low endogenous HSF activity and the long distances involved in axonal transport of *de novo* synthesised proteins. The source of the endogenous Hsp72 was found to be adjacent glia cells or other extra-neuronal sources, which synthesise and release a variety of molecules including that of the inducible form of Hsp72 into the extracellular environment. Here inducible Hsp72 and other released proteins are capable of generating effective stress

protein-mediated resistance to physical and metabolic insults. It is possible that several other cell types in the body may benefit from this type of protection *in vivo*, including human erythrocytes potentially from neighbouring leukocytes, endothelial cells or other cell types. The phenomenon of *ex vivo* human erythrocyte HSP based protection was shown to have physiological significance, as it was also demonstrated that erythrocyte fragility was decreased by immersion of the hands or fingers of healthy volunteers in hot water baths at febrile temperatures. This change in fragility of erythrocytes was achieved within ten minutes and required a temperature of more than 39°C, and was associated with the appearance of Hsp72 on the surface of the (non-permeabilized) human erythrocytes.

The erythrocytes of fish for example the rainbow and brook trout and the juvenile Atlantic salmon, like those of other non-mammalian vertebrates, are nucleated, retain their mitochondria and other cytoplasmic organelles and, in common with most cells have high levels of aerobic metabolism which supports many cellular processes including protein synthesis producing Hsp72 in response to elevated temperature stress (Currie & Tufts, 1997; Lund et al. 2003; Zarate & Bradley, 2003). This is presumably a protective mechanism allowing the cells to survive a stressful event, minimising the potentially detrimental effects induced by a stressful event and maintaining the cellular machinery in a serviceable state until conditions improve. Mature mammalian erythrocytes lack a nuclei and organelles and consequently do not contain DNA nor can they synthesise RNA and thus cannot respond in the same way. Hsp72 has been reported to be elevated internally and on the membrane of erythrocytes in patients with haemolytic anaemia's (Zarić et al. 1998) and malarial infection (Gudi & Gupta, 1993). It is unclear whether this Hsp72 is the result of stress during the development of the erythrocyte in the form of mutated (malfolded) proteins resulting in an altered erythrocyte membrane or is recruited in the way described here. Patients with aplastic anaemia have an exaggerated heat shock response in terms of Hsp72 production (Takami et al. 1999), and in sickle cell anaemia there is an increase in serum Hsp72 following a vaso-occlusive crisis (Adewoye et al. 2005). The association of anaemia with elevated Hsp72 responses suggests that the protein may have a role in maintaining membrane stability in these conditions and emphasises the importance of understanding the interaction with the erythrocyte

membrane. Furthermore, normal erythrocyte cytosol has also been demonstrated to possess the constitutive Hsc70 isoform and traceable amounts of the inducible isoform Hsp72 which was proposed to protect these cells from stressful conditions (Gudi & Gupta, 1993; Zarić et al. 1998). In addition, antibodies to Hsp60 and Hsp10 have been found in mature erythrocytes, these observations may in fact be possible due to the events occurring in erythrocyte maturation where all organelles are extruded, erythrocytes may retain mitochondrial proteins that may be required for particular functions (Alberts et al. 2002). The data in this chapter is in agreement with these previous findings (Gudi & Gupta, 1993; Zarić et al. 1998) as seen during the experiments (Figures 5.8-5.12 and Table 5.1) where bovine Hsp70 or recombinant human Hsp72 were added to isolated human erythrocytes. The protective effects of exogenously administered HSPs are proposed to be through binding to the cell surface of non-permeabilized cells and/or internalisation (in permeabilized cells) and may in some form block or interfere with events leading to haemolysis. The data shows binding of extracellular HSPs to the cell surface on non-permeabilized cells took less than an hour. In fact, Hsp72 bound rapidly to the erythrocyte surface within twenty minutes of incubation with 10 µg/mL Hsp72 and binding increased until one hour where a plateau was reached. A significant uptake and internalisation of Hsp72 by permeabilized erythrocytes was also detectable at this time point (twenty minutes), and increased up to eighty minutes, increasing the erythrocytes total internal HSP content significantly compared to non-permeabilized cells as determined by flow cytometry. These results were confirmed by fluorescence microscopy, and the images showed rapid staining of the erythrocyte membrane. However, not all of the whole cell population (23 %) was able to bind or internalise Hsp72 (determined by flow cytometry), which may be due to the age of the cell and the optimum lifespan for effectively binding and/or internalising protein. To verify that the administered bovine Hsp70 or human Hsp72 was bound to the erythrocyte surface and/or taken up, the presence of Hsp72 within the membrane or inside erythrocytes was detected by Western blotting. The results found the protein to be associated with the cell membrane but it was not detected in the cytosol. This may be because the protein in permeabilized cells is associated/incorporated within the inner side of the membrane and so not detectable inside the cells. However, it may be speculated after a longer length of

incubation (ninety minutes) with human Hsp72 (as seen in the flow cytometry data), the protein may dissociate from the membrane and possibly enter the cytosol, this would require further investigation. Thus, Hsp72 is taken up by the enucleate erythrocytes providing protection from conditions such as *in vivo* metabolic starvation and fever. Monocytes and neuronal cells have also been shown to take up extracellular Hsp70 and are then resistant to heat stress and TNF- $\alpha$  apoptosis (Guzhova et al. 1998; Guzhova et al. 2001), despite the fact that monocytes are capable of generating their own Hsp72. In fact, many cells will bind and internalise Hsp72 when presented with it *in vitro* (Guzhova et al. 1998; Guzhova et al. 2001). However, this chapter has shown that, as in the case of the aortic smooth muscle cells (Johnson et al. 1990), internalisation of the Hsp72 protein is not required for protection. This further supports the suggestion that Hsp72 is stabilising the lipid membrane.

#### **5.4.2 Receptor mediated protection: SR-A1.**

The protective effects of Hsp72 introduced into human erythrocytes can be potentially explained by an interaction with certain receptors on the cell surface. Hsp72 and Hsc70 can co-localise with the membrane lipid PS followed by rapid incorporation into the lipid bilayer (Arispe et al. 2004). PS should only be present on the surface of damaged/apoptotic or senescent erythrocytes and is not detectable in a normal population (Walsh et al. 2002). Two experiments suggest that PS is not responsible for the Hsp72 interaction with erythrocytes. Firstly, PS was not detected on the surface of healthy human erythrocytes using Annexin V by flow cytometry. Secondly, Annexin V did not inhibit either Hsp72 binding to erythrocytes, or the Hsp72 mediated decrease in erythrocyte fragility. It is therefore likely that the internalisation is receptor mediated which is supported by the kinetic data. Hsp72 has been reported to interact with a number of receptors on cells including CD91 on macrophages and DCs (Binder et al. 2000; Basu et al. 2001), the TLR family including TLR-2 and TLR-4 with their cofactor CD14 on monocytes (Asea et al. 2000b; Asea et al. 2002), CD40, a member of the TNF receptor family on B cells (Becker et al. 2002) and LOX-1 type SRs on endothelial cells (Delneste et al. 2002; Thériault et al. 2005; Thériault et al. 2006). Hsp72 binding to erythrocytes, and the resulting decrease in membrane fragility, was inhibited by malBSA, a general SR ligand (Takata et

al. 1989; Sakai et al. 1996), suggesting involvement of SRs. Inhibition of Hsp72 binding, and decreasing fragility, was achieved with the SR-A type receptor ligands (fucoidan and Poly (I)), but not with an anti-LOX-1 antibody or with an anti-CD77 antibody which detects Gb3. It is therefore proposed that Hsp72 is binding to SR-A1 on the erythrocyte membrane, prior to a possible internalisation, the presence of which was confirmed by several methods: flow cytometry, fluorescence microscopy and Western blotting. This interaction was shown to be essential for cell membrane stability and protection. The data in this chapter supports the hypothesis that erythrocytes become resistant to potentially lethal trauma when supplemented by the administration of exogenous Hsp72. Indeed, oxidative stress which is experienced in type-1 and type-2 diabetes results in a reduced erythrocyte lifespan (Leoncini et al. 2008). These patients typically have reduced expression of Hsp72 (Chung et al. 2008) and would therefore be limited in their ability to respond in the manner described here. Here it may be predicted that treatments used by Chung et al. (2008) to increase Hsp72 expression would also result in restoration of erythrocyte longevity in diabetic patients.

The data presented in this chapter is novel, it is the first to show enucleate cells, namely human erythrocytes can bind and take up Hsp72, resulting in the protection it offers to nucleate cells that synthesise this protein in response to conditions of stress. The data demonstrates that exogenous Hsp72 is able to confer stress tolerance to a cell type which is unable to synthesise Hsp72. This study is also the first to identify SR-A1 receptors on erythrocyte membranes and suggests a dual role for this type of receptor. Thus, it can be concluded that Hsp72 has a biological role in the protection of human erythrocytes from physiological stress. Previous studies (Guzhova et al. 1998; Guzhova et al. 2001) rely on cultured cells to demonstrate the effects of extracellular Hsp72, whereas the investigations in this chapter use primary cells *in vitro* and also demonstrate functional relevance *in vivo*. The cyto-protective effects of extracellular Hsp72 may be of potential interest in the future for the therapies of traumas or other injuries and therefore more in-depth studies are required to understand this potential effect. Further work will investigate the mechanism(s) by which

extracellular Hsp72 exerts its beneficial effects and the role of SRs in erythrocyte biology.

## Chapter 6

### **Extracellular Hsp72 activates macrophage differentiation, stimulates cytokines, and is a chemoattractant for macrophages and human leukocytes.**

#### **6.1 Introduction**

The previous chapter determined that extracellular recombinant human Hsp72 is able to provide protection of erythrocytes from physiological and environmental stresses through interaction with SRs, such as SR-A1 on the cell surface. Several other roles for extracellular HSPs have been proposed (Matzinger, 2002; Ménoret et al. 2002; Campisi & Fleshner, 2003a; Campisi et al. 2003b), including immune activation. HSPs are reported to be highly immunogenic, and extracellular Hsp72 released from damaged, injured or necrotic cells (Basu et al. 2000b; Shi et al. 2000; Shi et al. 2002), has been postulated to act as an endogenous pro-inflammatory stimulus for the innate immune response (Asea et al. 2000b; Matzinger, 2002; Campisi & Fleshner, 2003a; Campisi et al. 2003b; Svensson et al. 2006). Extracellular Hsp72 facilitates innate immunity and promotes local inflammation during pathological events through activation of APCs (Guzhova et al. 1998; Gallucci et al. 1999; Asea et al. 2000b; Basu et al. 2000b) and stimulation of both pro- and anti-inflammatory cytokines (Basu et al. 2000b; Asea et al. 2000b; Svensson et al. 2006), chemokines (Lehner et al. 2000) and NO release (Panjwani et al. 2002) *via* high-affinity receptor mediated processes (Thériault et al. 2005; Thériault et al. 2006). The ability of extracellular Hsp72 to activate the innate immune system suggests that it has properties consistent with a role as a ‘danger’ signalling molecule to immune cells, improving host defence and maintaining homeostasis (Matzinger, 1994), through its ability to up-regulate co-stimulatory surface molecules CD40, CD83 and CD86 (Banchereau et al. 2000) and the release of IL-12 from naïve DCs (Bausero et al. 2005). Several workers have demonstrated the stimulation and release of cytokines by extracellular Hsp72, including Campisi et al. (2003b) who found Hsp72 could stimulate TNF- $\alpha$ , IL-1 $\beta$  and IL-6 secretion from macrophages. Also Svensson et al. (2006) observed a 3-fold increase of IL-1 $\beta$  and IL-12 in naïve macrophages when stimulated with supernatant from PBMCs



containing Hsp72 and more recently Vega et al. (2008) demonstrated that Hsp72 could activate macrophages when in a membrane bound form.

There are three main types of professional APCs, these include DCs, macrophages and B cells and this chapter will focus on macrophages. Macrophages first termed by Metchnikoff in 1892, are key players in innate and adaptive immunity to foreign invaders such as infectious microorganisms, as well as in the inflammatory response. During the differentiation of pro-monocytes to monocytes and macrophages it has been shown that there is an increase in the expression of several cell surface receptors. These include CD11a/CD18, CD11b/CD18, CD14, CD68, CD163 and SRs (Huh et al. 1995; Guzhova et al. 1998; Panjwani et al. 2002; Daigneault et al. 2010), which are involved in the recognition and phagocytosis of bacteria, fungi and apoptotic cells (Platt et al. 1996; Armesilla et al. 1996; Panjwani et al. 2002; Maniecki et al. 2006; Murphy et al. 2005), suggesting their involvement within innate immunity (Krieger & Hertz, 1994).

### **6.1.1 Aims**

The aims of this chapter are to investigate: whether extracellularly administered (bovine Hsp70 or human Hsp72); intracellular (heat-induced); released extracellular HSP; or HSP enriched (heat treated intracellular) supernatant is able to stimulate an immune response in U937 macrophages and human leukocytes.

## **6.2 Methods**

All preparations and cell culture experiments were performed using aseptic technique in a class II tissue culture hood.

### **6.2.1 Preparation of cells for experimental treatments.**

U937 macrophages were prepared as in section 2.3.6. Human monocytes were isolated and prepared for treatment as in section 2.3.10.

### **6.2.2 Preparation of HSPs for experimental treatments.**

HSPs used for experiments were: bovine Hsp70 or recombinant human Hsp72. Stock solutions used were 0.5 mg/mL bovine Hsp70 in dH<sub>2</sub>O and 0.1 mg/mL human Hsp72 in PBS.

### **6.2.3 Treatment of cells with phorbol 12-myristate 13-acetate (PMA).**

PMA was made-up to the appropriate concentrations of 20, 10, 1 or 0 ng/mL in 10 % (v/v) HI-RPMI before being applied to cells for 0-48 hours at 37°C.

### **6.2.4 Pre-treatment of cells with bovine Hsp70 or human Hsp72.**

Bovine Hsp70 and human Hsp72 were made-up to the appropriate concentrations of 100, 50, 10, 1 or 0 µg/mL in 10 % (v/v) HI-RPMI before being applied to cells for 1 hour at 37°C before further treatments.

### **6.2.5 Time course experiments with human Hsp72**

Human Hsp72 was applied to cells at 1:10 dilution (100 µL protein stock to 900 µL of 10 % (v/v) HI-RPMI) so that the cells were treated with 10 µg/mL Hsp72. Cells were then incubated for up to 48 hours at 37°C.

### **6.2.6 Heat shock treatment of cells.**

The heat shock experiments were performed using a temperature-controlled stirred water bath set to 37°C, 42°C or 46°C for 1 hour. Cells were then transferred to a 37°C incubator with 5 % CO<sub>2</sub> for 1 hour recovery before further treatments.

### **6.2.7 Treatment of cells with Hsp72 enriched supernatant.**

U937 monocytes (in 10 % (v/v) HI-RPMI) were heated in a temperature-controlled stirred water bath set to 37°C, 42°C or 46°C for 1 hour, followed by 1 hour recovery at 37°C. Cells were then centrifuged at 400 g for 5 minutes at 25°C and the supernatant was removed and transferred to clean 15 mL centrifuge tubes before being applied to cells for 3 hours at 37°C. The cell pellet was also re-suspended in 10 mL 10 % (v/v) HI-RPMI, before being applied to cells for 3 hours at 37°C.

### **6.2.8 Treatment of cells with anti-human Hsp72.**

Anti-human Hsp72 was diluted at 1:500, 1:250, 1:100, 1:50 or 1:10 in 10 % (v/v) HI-RPMI and incubated with the Hsp72 enriched supernatant for 1 hour before being applied to cells for 3 hours at 37°C.

### **6.2.9 Treatment of cells with Brij®-98.**

Brij®-98 was made-up to the appropriate concentrations of 0.5 or 0 % in 10 % (v/v) HI-RPMI and incubated with the Hsp72 enriched supernatant for 10 minutes at 4°C before being applied to cells for 3 hours at 37°C.

### **6.2.10 Measurement of surface markers: CD36, SR-A1 and CD91.**

Flow cytometry was used to measure CD36, SR-A1 and CD91 on the cell surface of U937 monocytes, U937 macrophages and human monocytes (Section 2.3.20). Cells were incubated with anti-CD36: FITC (1:250 dilution), anti-SR-A1 (1:50 dilution) with a secondary IgG R-PE conjugated antibody (1:50 dilution), or anti-CD91: FITC (1:250 dilution), 50 µL/sample for 40 minutes at 4°C in the dark.

### **6.2.11 Measurement of secreted cytokines or Hsp72.**

Supernatant was removed from the wells and centrifuged at 400 g for 5 minutes at 25°C and then transferred to clean 1.5 mL microcentrifuge tubes. Supernatant was then either used fresh or frozen at -70°C until required. Secretion of TNF- $\alpha$ , IL-10 and Hsp72 were measured as described in sections 2.3.39-2.3.41.

#### **6.2.12 Effect on migration of U937 macrophages and human leukocytes by extracellular Hsp72.**

Migration of U937 macrophages and human leukocytes was determined as in section 2.3.28.

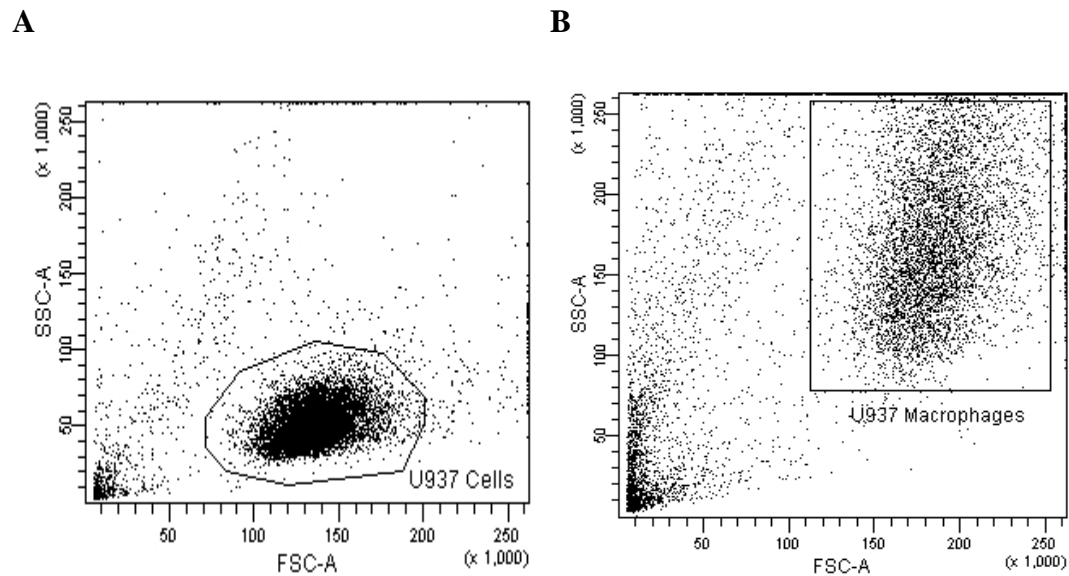
200  $\mu$ L of human Hsp72 (0-100  $\mu$ g/mL in 10 % HI-RPMI) or Hsp72 enriched supernatant was added to the lower chamber of a HTS Transwell® permeable support system for 4 hours (U937 macrophages) or 2 hours (Human leukocytes). LPS (100 ng/mL) in 10 % HI-RPMI, FMLP (10 nM) in 10 % HI-RPMI or 10 % HI-RPMI were used as controls.

#### **6.2.11 Cell counts and viability tests by trypan blue exclusion.**

Following treatment, cells were re-suspended into the media by reflux pipetting. Migrated cells were then counted and viability tested using trypan blue exclusion assay as described in section 2.4.3.

### 6.3 Results

The first part of the study was to analyse the cell surface expression of CD36, SR-A1 and CD91 on U937 monocytes and U937 macrophages under control conditions (1 hour culture at 37°C), and following incubation with PMA (10 ng/mL, 48 hours at 37°C). U937 monocytes and U937 macrophage populations were distinguished using FSC/SSC (Figure 6.1) and the cell surface expression of CD36, SR-A1 and CD91 by flow cytometry (Figures 6.2-6.4).



**Figure 6.1: Identification of U937 monocytes (A) and U937 macrophages (B) using FSC/SSC on the flow cytometer.**

$5 \times 10^5$  cells/mL U937 monocytes and U937 macrophages were gated using FSC/SSC respectively and 10,000 events were recorded.

### **6.3.1 CD36, SR-A1 and CD91 surface expression on U937 monocytes and macrophages following incubation with PMA.**

The main objective of this part of the study was to investigate the surface expression of CD36, SR-A1 and CD91 under normal conditions and to examine the changes in surface expression in response to treatment with PMA over a period of forty-eight hours (Figures 6.2-6.4).

#### **6.3.1.1 Surface expression of CD36, SR-A1 and CD91 on control U937 monocytes.**

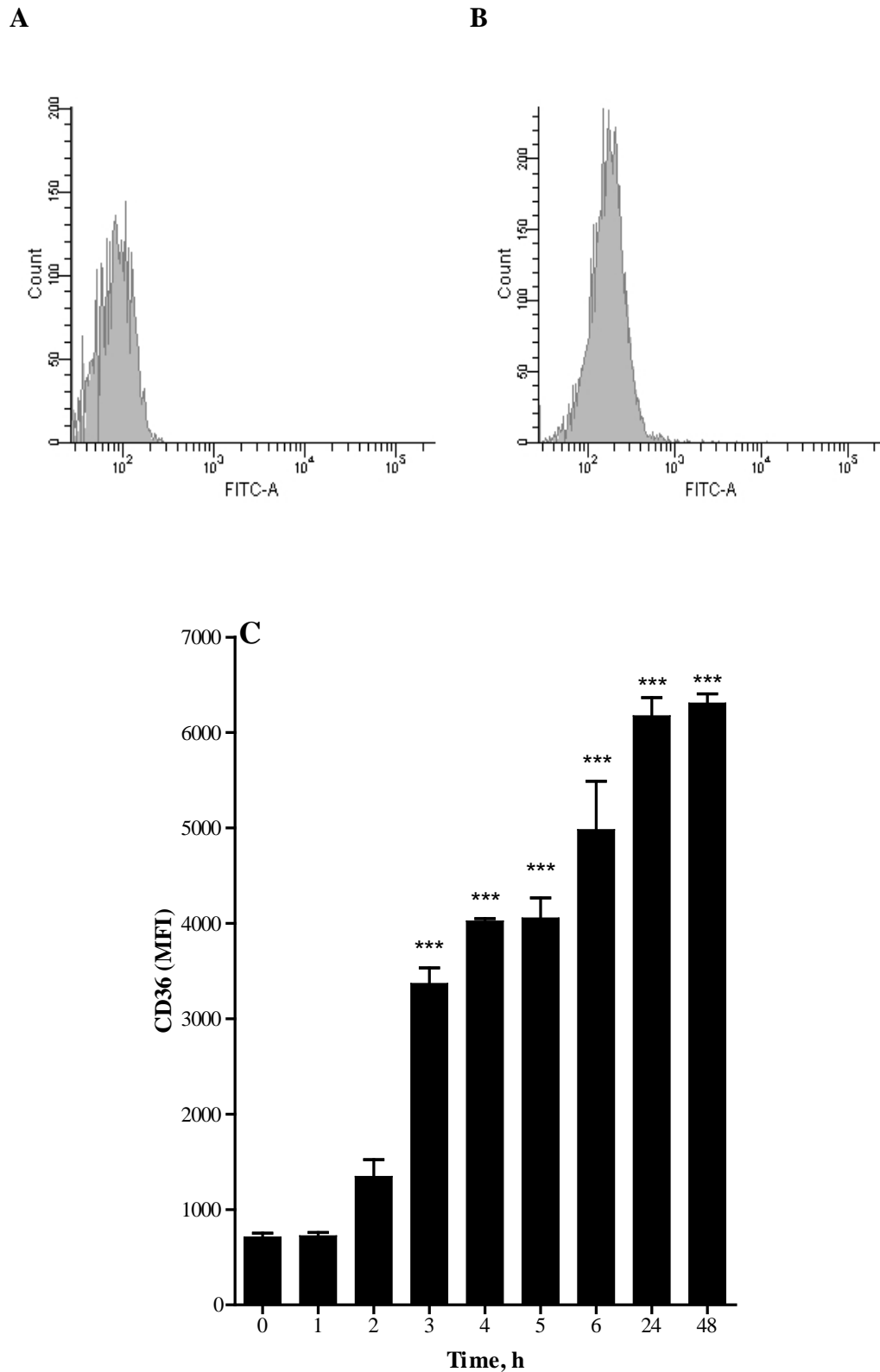
The initial experiments shown in the histograms and bar charts of figures 6.2-6.4 suggest U937 monocytes to express low base line levels of CD36, SR-A1 and CD91 on their cell surface under control conditions (37°C). The results shown in figure 6.2C reveal the base line level of CD36 to be significantly greater than surface expressed SR-A1 ( $P<0.05$ , Figures 6.2-6.3C) and CD91 ( $P<0.001$ , Figures 6.2-6.4C). Also, the results presented in figure 6.3C reveal the base line level of SR-A1 to be significantly greater than CD91 ( $P<0.01$ , Figures 6.3-6.4C), suggesting U937 monocytes express significantly increased levels of both CD36 ( $P<0.001$ ) and SR-A1 ( $P<0.01$ ) on their cell surface compared to CD91 under control conditions.

#### **6.3.1.2 Surface expression of CD36, SR-A1 and CD91 on U937 monocytes and macrophages following treatment with PMA.**

Following incubation of U937 monocytes with PMA for up to forty-eight hours, an almost 5-fold increase ( $P<0.001$ ) of CD36 surface expression was seen at three hours compared to control cells (Figure 6.2C). At incubation times below this (one–two hour) there was no significant difference from control (Figure 6.2C). As the length of exposure to PMA increased, the surface expression of CD36 increased up to twenty-four hours compared to control ( $P<0.001$ , Figure 6.2C) and reached a plateau of expression at forty-eight hours ( $P<0.001$ , Figure 6.2C). A 4-fold increase ( $P<0.05$ ) of SR-A1 surface expression was also observed with PMA but after a six hour exposure compared to control cells (Figure 6.3C). At incubation times below this (one-five hour) there was no significant difference from control (Figure 6.3C). Again, as the length of exposure to PMA increased, the surface expression of SR-A1 increased up to twenty-four hours compared to

control ( $P<0.001$ , Figure 6.3C), and in contrast to CD36 expression increased significantly up to forty-eight hours to reach an almost 22-fold increase compared to control cells ( $P<0.001$ , Figure 6.3C). Furthermore, a significant increase of CD91 surface expression was also seen with PMA, however this was not observed until after twenty-four hour exposure, where a 7-fold increase ( $P<0.001$ ) was seen compared to control (Figure 6.4C), and which increased up to forty-eight hours to reach an almost 14-fold increase compared to control cells ( $P<0.001$ , Figure 6.4C).

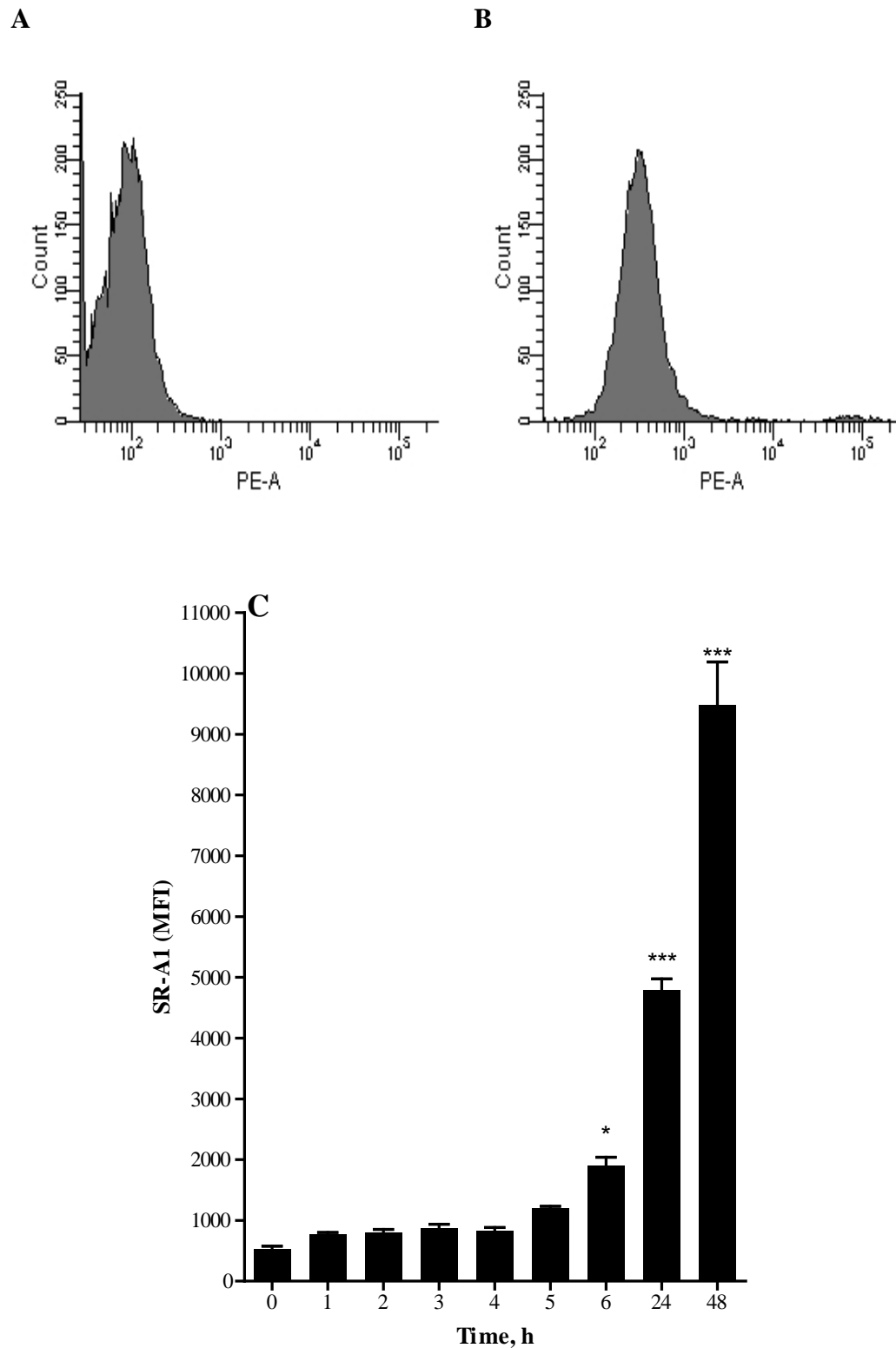
Under control conditions U937 monocytes were found to express significant levels of CD36 compared to both SR-A1 ( $P<0.05$ ) and CD91 ( $P<0.001$ ), which increased with PMA treatment ( $P<0.001$ , Figure 6.2C). However, following the exposure of U937 monocytes to PMA for forty-eight hours revealed an alteration in the cell surface expression of CD36, SR-A1 and CD91. U937 macrophages were found to express a significantly increased level of SR-A1 compared to both CD36 ( $P<0.01$ , Figure 6.2C) and CD91 ( $P<0.001$ , Figure 6.4C).



**Figure 6.2: The effect of PMA (10 ng/mL, 0-48 hours, 37°C) on the expression of CD36 on U937 monocytes and macrophages.**

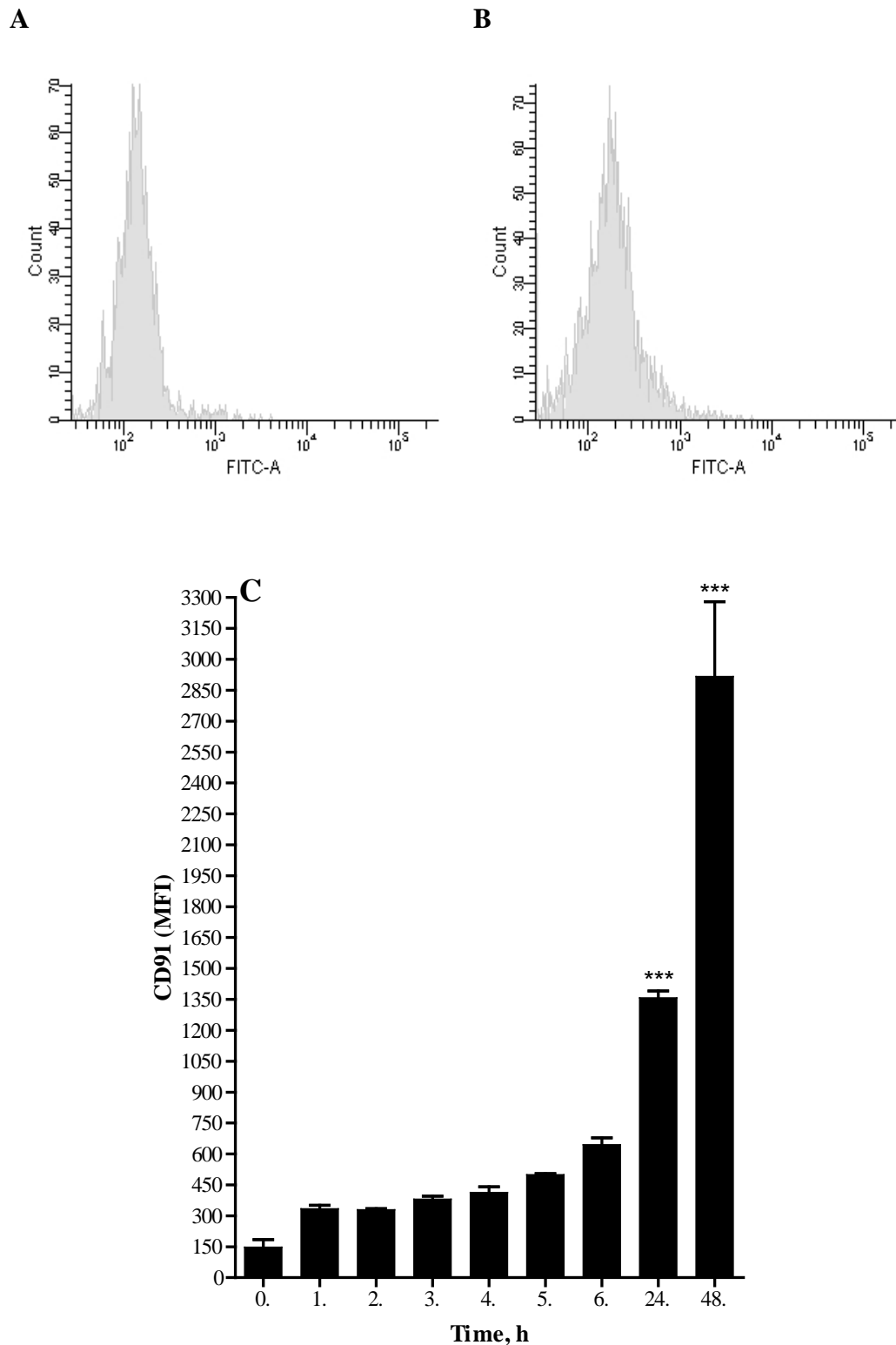
(A) No stain control sample. (B) CD36 expression at 37°C. (C) CD36 expression over forty-eight hours. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean time points from 0 hour through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).





**Figure 6.3: The effect of PMA (10 ng/mL, 0-48 hours, 37°C) on the expression of SR-A1 on U937 monocytes and macrophages.**

(A) No stain control sample. (B) SR-A1 expression at 37°C. (C) SR-A1 expression over forty-eight hours. Data are presented mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean time points from 0 hour through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\*\* ( $P<0.001$ ).



**Figure 6.4: The effect of PMA (10 ng/mL, 0-48 hours, 37°C) on the expression of CD91 on U937 monocytes and macrophages.**

(A) No stain control sample. (B) CD91 expression at 37°C. (C) CD91 expression over forty-eight hours. Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean time points from 0 hour through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).

### **6.3.2 Treatment of U937 monocytes with bovine Hsp70 or human Hsp72 and/or PMA.**

The results of the previous experiments confirmed that the pattern of expression of the surface markers CD36, SR-A1 and CD91 are suitable indicators of U937 monocyte differentiation into U937 macrophage. Visual confirmation of monocyte differentiation can also be observed in the images presented in figure 6.10. The aims of the following experiments were to investigate the effect of bovine Hsp70 or human Hsp72 (0-100  $\mu\text{g/mL}$ , one hour) on monocyte differentiation compared to PMA and also to examine the effect of bovine Hsp70 or human Hsp72 in combination with PMA on monocyte differentiation.

Surface expression of CD36, SR-A1 and CD91 on U937 monocytes in response to incubation with bovine Hsp70 or human Hsp72 and/or PMA are presented in Figures 6.5-6.9.

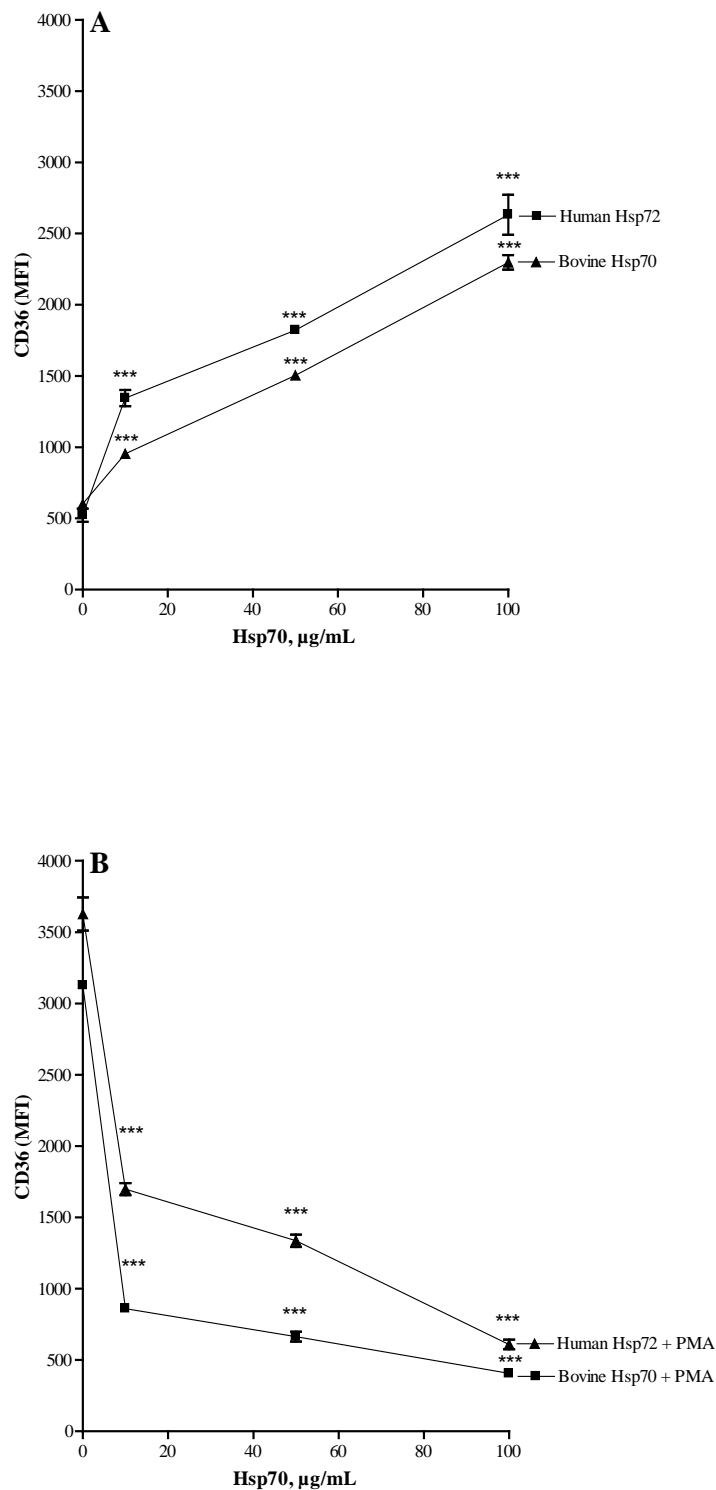
#### **6.3.2.1 Surface expression of CD36 on U937 monocytes following a pre-treatment with bovine Hsp70 or human Hsp72 and/or PMA.**

Following incubation of U937 monocytes with bovine Hsp70 or human Hsp72 for one hour led to a significant increase in the surface expression of CD36 compared to control cells ( $P < 0.001$ , Figure 6.5A). An almost 3-fold increase ( $P < 0.001$ ) was seen with 10  $\mu\text{g/mL}$  human Hsp72 and a 2-fold increase ( $P < 0.001$ ) with 10  $\mu\text{g/mL}$  bovine Hsp70 compared to control cells (Figure 6.5A). The results revealed as the concentration of HSP increased, the surface expression of CD36 increased, 4-fold ( $P < 0.001$ ) with 50  $\mu\text{g/mL}$  human Hsp72 and 3-fold ( $P < 0.001$ ) with 50  $\mu\text{g/mL}$  bovine Hsp70 compared to control cells (Figure 6.5A). CD36 expression also increased almost 6-fold ( $P < 0.001$ ) with the highest concentration of 100  $\mu\text{g/mL}$  human Hsp72 and almost 4-fold ( $P < 0.001$ ) with 100  $\mu\text{g/mL}$  bovine Hsp70 compared to control cells (Figure 6.5A). Human Hsp72 was found to be more effective than bovine Hsp70 at increasing the surface expression of CD36 after a one hour incubation at concentrations of 10  $\mu\text{g/mL}$  ( $P < 0.01$ ), 50  $\mu\text{g/mL}$  ( $P < 0.01$ ) and 100  $\mu\text{g/mL}$  ( $P < 0.01$ , Figure 6.5A). In contrast, the addition of bovine Hsp70 or human Hsp72 for one hour prior to incubation with PMA for three hours led to a significant decrease in the surface expression of CD36 compared to control cells ( $P < 0.001$ , Figure 6.5B). CD36

expression decreased 2-fold ( $P<0.001$ ) when incubated with 10  $\mu\text{g/mL}$  human Hsp72 and 3-fold ( $P<0.001$ ) with 10  $\mu\text{g/mL}$  bovine Hsp70 compared to control cells (Figure 6.5B). CD36 surface expression also decreased over 2-fold ( $P<0.001$ ) with 50  $\mu\text{g/mL}$  human Hsp72 and almost 4-fold ( $P<0.001$ ) with 50  $\mu\text{g/mL}$  bovine Hsp70 compared to control cells (Figure 6.5B). Similarly, with the increased concentration of 100  $\mu\text{g/mL}$  HSP, CD36 expression decreased significantly ( $P<0.001$ , Figure 6.5B) to almost 7-fold ( $P<0.001$ ) with human Hsp72 and 8-fold ( $P<0.001$ ) with bovine Hsp70 compared to control cells (Figure 6.5B). The results suggest bovine Hsp70 to be less effective than human Hsp72 at increasing the surface expression of CD36 after a one hour incubation at concentrations of 10  $\mu\text{g/mL}$  ( $P<0.001$ ) and 50  $\mu\text{g/mL}$  ( $P<0.001$ ) before PMA treatment, however there was no significant difference at 100  $\mu\text{g/mL}$  HSP (Figure 6.5A).

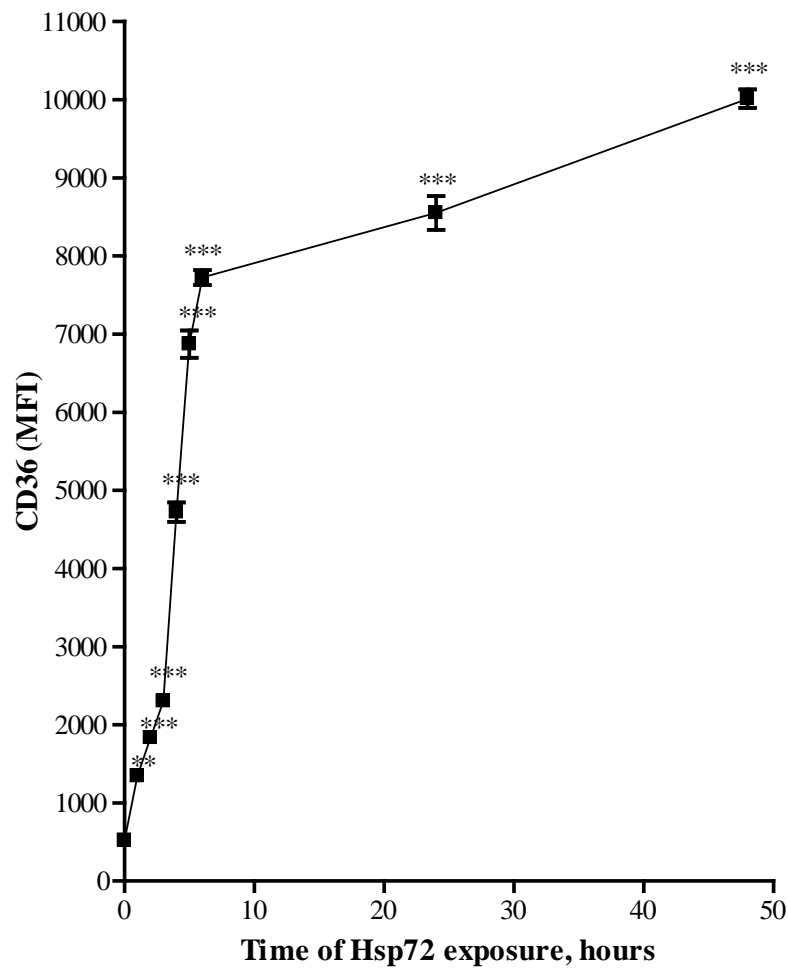
#### **6.3.2.2 Time course of CD36 surface expression on U937 monocytes and macrophages following treatment with human Hsp72.**

Incubation of U937 monocytes with human Hsp72 (10  $\mu\text{g/mL}$ ) for up to forty-eight hours was found to lead to a significant increase in the surface expression of CD36 compared to control cells ( $P<0.01$  to  $P<0.001$ , Figure 6.6). Consistent with previous findings (Figure 6.5), an almost 3-fold increase ( $P<0.01$ ) of CD36 expression was seen after a one hour incubation with human Hsp72 compared to control cells (Figure 6.6). These results are in contrast to those obtained with PMA where a significant effect on CD36 expression was not observed until three hour incubation (Figure 6.2C). The results revealed as the exposure time to human Hsp72 increased, the surface expression of CD36 increased, 14-fold ( $P<0.001$ ) after six hours, almost 15-fold ( $P<0.001$ ) after twenty-four hours and in contrast to PMA where a plateau of expression was seen (Figure 6.2C), a 17-fold increase after forty-eight hours ( $P<0.001$ , Figure 6.6) compared to control cells. These results suggest human Hsp72 to be more effective than PMA at increasing the surface expression of CD36 on U937 monocytes and U937 macrophages after a forty-eight hour incubation at a concentration of 10  $\mu\text{g/mL}$  ( $P<0.001$ , Figure 6.6).



**Figure 6.5: The effect of 1 hour incubation with 0-100  $\mu\text{g/mL}$  bovine Hsp70 or human Hsp72 (A) and/or 3 hour incubation with 10  $\text{ng/mL}$  PMA (B) at  $37^\circ\text{C}$  on the expression of CD36 on U937 monocytes.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . \* indicates significant difference between mean concentrations from 0  $\mu\text{g/mL}$  through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



**Figure 6.6: The effect of human Hsp72 (10  $\mu\text{g/mL}$ , 0-48 hours, 37°C) on the expression of CD36 on U937 monocytes and macrophages.**

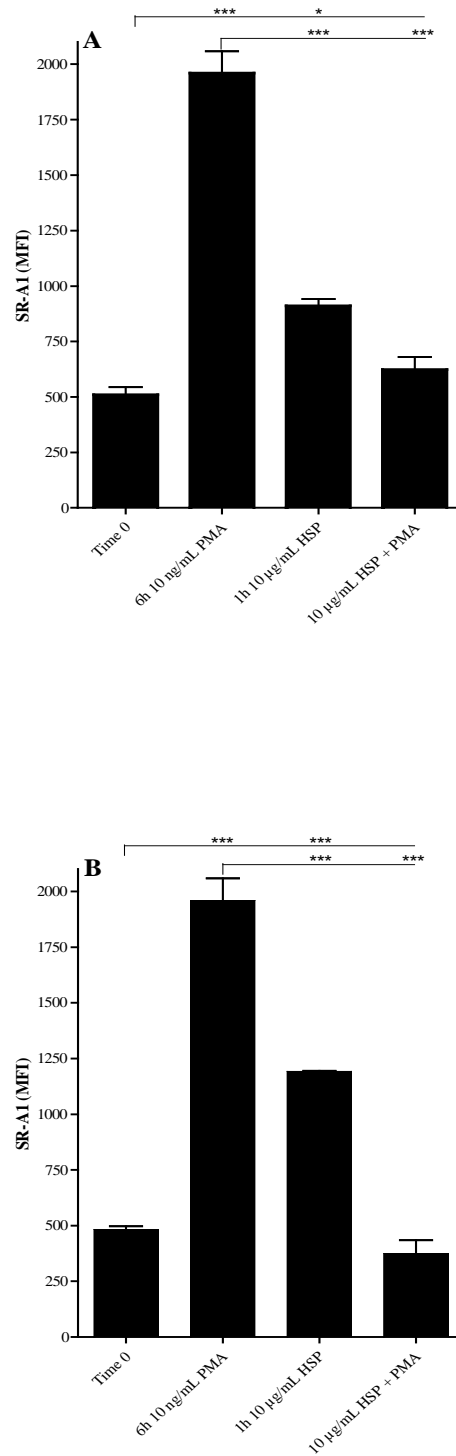
Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference from 0 hour using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).

### **6.3.2.3 Surface expression of SR-A1 on U937 monocytes following a pre-treatment with bovine Hsp70 or human Hsp72 and/or PMA.**

The results shown in figures 6.5A-B and 6.6 revealed significant differences with CD36 expression using the lowest concentration of HSP, 10 µg/mL and thus will be used throughout the following experiments (Figures 6.6-6.8).

Similar to the experiments investigating the surface expression of CD36, incubation of U937 monocytes with bovine Hsp70 or human Hsp72 for one hour led to a significant increase in the surface expression of SR-A1 compared to control cells ( $P < 0.05$  to  $P < 0.001$ , Figure 6.7A-B). An almost 2-fold increase ( $P < 0.05$ , Figure 6.7A) was seen with 10 µg/mL bovine Hsp70 and an almost 3-fold increase ( $P < 0.001$ , Figure 6.7B) with 10 µg/mL human Hsp72 compared to control cells (Figure 6.7A-B). Similarly, treatment with PMA for six hours also led to a significant, 5-fold increase ( $P < 0.001$ ) in the surface expression of SR-A1 compared to control cells (Figure 6.7A-B), which are consistent with previous findings (Figure 6.3). Human Hsp72 was found to be more effective than bovine Hsp70 at increasing the surface expression of SR-A1 after a one hour incubation with 10 µg/mL HSP ( $P < 0.05$ , Figure 6.7A-B).

In comparison, the addition of bovine Hsp70 or human Hsp72 for one hour prior to incubation with PMA for six hours led to a decrease in the surface expression of SR-A1 compared to control cells and to the PMA control (Figure 6.7A-B). SR-A1 expression decreased 3-fold ( $P < 0.001$ ) with 10 µg/mL bovine Hsp70 (Figure 6.7A) and 8-fold ( $P < 0.001$ ) with 10 µg/mL human Hsp72 (Figure 6.7B) compared to the PMA control. However, compared to control cells the decrease in SR-A1 surface expression was not significant with both 10 µg/mL, bovine Hsp70 or human Hsp72 (Figure 6.7A-B). The results suggest human Hsp72 to be more effective than bovine Hsp70 at not increasing the surface expression of SR-A1 after a one hour incubation with a concentration of 10 µg/mL HSP ( $P < 0.01$ , Figures 6.7A-B) before PMA treatment, which is in contrast to the results observed with CD36.



**Figure 6.7: The effect of 1 hour 10 µg/mL bovine Hsp70 (A) or human Hsp72 (B) at 37°C on PMA induced expression of SR-A1 on U937 monocytes.**

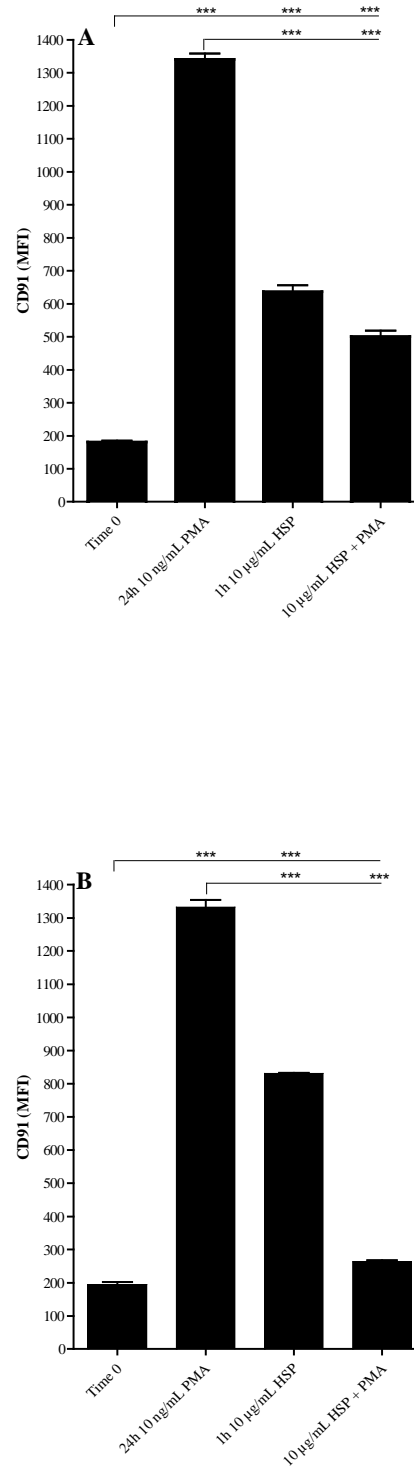
Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean treatments from time 0 control or PMA control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\*\* (P<0.001).



**6.3.2.4 Surface expression of CD91 on U937 monocytes following a pre-treatment with bovine Hsp70 or human Hsp72 and/or PMA.**

As with the expression of both surface CD36 and SR-A1, incubation of U937 monocytes with bovine Hsp70 or human Hsp72 for one hour led to a significant increase in the surface expression of CD91 compared to control cells (Figure 6.8A-B). An almost 3-fold increase ( $P<0.001$ , Figure 6.8A) was seen with 10  $\mu\text{g/mL}$  bovine Hsp70 and an almost 4-fold increase ( $P<0.001$ , Figure 6.8B) with 10  $\mu\text{g/mL}$  human Hsp72 compared to control cells (Figures 6.8A-B). In addition, treatment of U937 monocytes with PMA for up to twenty-four hours also led to a significant, 6-fold increase ( $P<0.001$ ) in the surface expression of CD91 compared to control cells (Figure 6.8A-B), which are consistent with previous findings (Figure 6.4). Similar to the expression of both CD36 and SR-A1, human Hsp72 was found to be more effective than bovine Hsp70 at increasing the surface expression of CD91 after a one hour incubation with 10  $\mu\text{g/mL}$  HSP ( $P<0.001$ , Figures 6.8A-B).

In contrast, the addition of bovine Hsp70 or human Hsp72 for one hour prior to incubation with PMA for twenty-four hours led to a decrease in the surface expression of CD91 compared to control cells and the PMA control (Figures 6.8A-B). CD91 expression decreased almost 3-fold ( $P<0.001$ ) compared to the PMA control and almost 1-fold ( $P<0.001$ ) compared to control cells with 10  $\mu\text{g/mL}$  bovine Hsp70 (Figure 6.8A). Similarly, an almost 6-fold decrease ( $P<0.001$ ) of CD91 expression was seen with 10  $\mu\text{g/mL}$  human Hsp72 compared to the PMA control, however compared to control cells the decrease was found to be not significant (Figure 6.8B). The results suggest human Hsp72 to be more effective than bovine Hsp70 at not increasing the surface expression of CD91 after a one hour incubation with a concentration of 10  $\mu\text{g/mL}$  HSP ( $P<0.001$ , Figures 6.8A-B) before PMA treatment, which are similar to the results obtained with SR-A1 but in contrast to those observed with CD36.

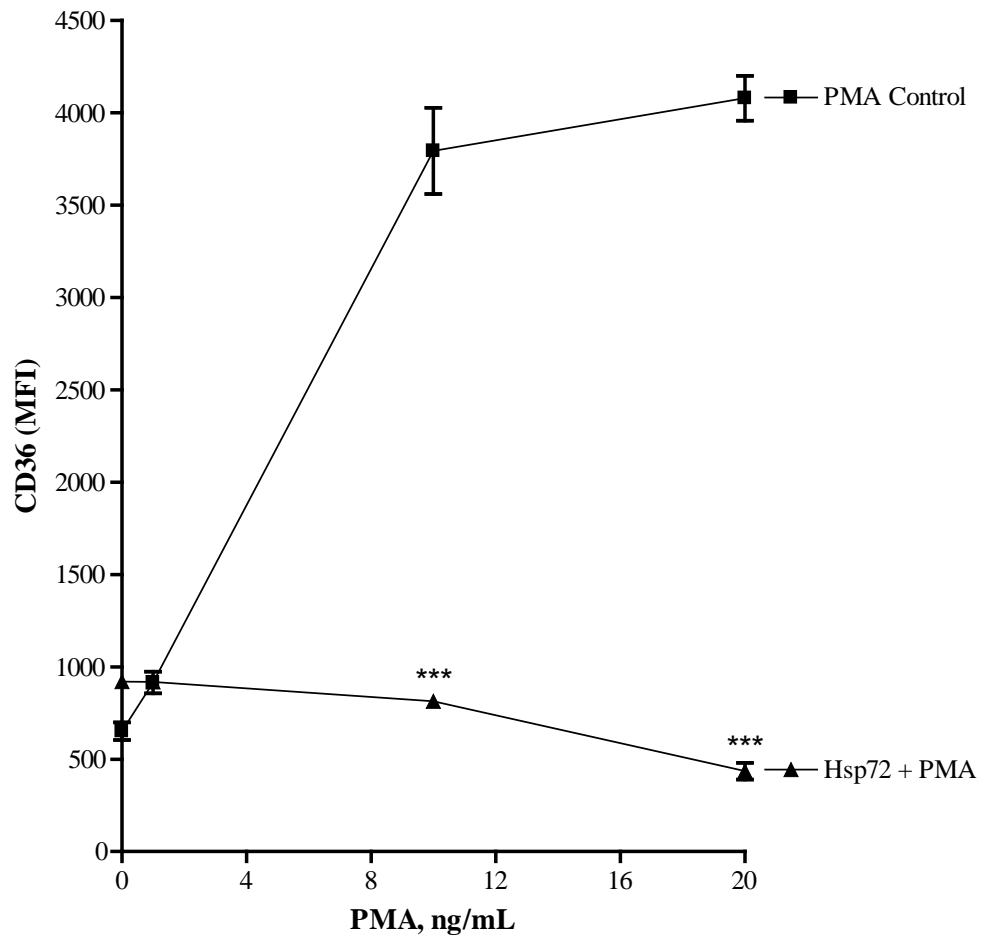


**Figure 6.8: The effect of 1 hour 10 µg/mL bovine Hsp70 (A) or human Hsp72 (B) at 37°C on PMA induced expression of CD91 on U937 monocytes.** Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean treatments from time 0 control or the PMA control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).

#### **6.3.2.5 Treatment of U937 monocytes with PMA and/or human Hsp72 and the effect on the surface expression of CD36.**

The following experiment was performed to determine whether the decrease in expression of the surface markers CD36, SR-A1 and CD91 on U937 monocytes and macrophages when incubated with HSP and PMA was due to a potential interaction with HSP and PMA.

Consistent with previous findings (Figure 6.2C), incubation of U937 monocytes with 10 ng/mL PMA for three hours led to a significant, an almost 5-fold increase ( $P<0.001$ ) in the surface expression of CD36 compared to control cells (Figure 6.9). At concentrations below this (1 ng/mL) there was no significant difference from control (Figure 6.9). As the concentration of PMA increased to 20 ng/mL, the surface expression of CD36 increased to reach a significant 7-fold increase compared to control cells ( $P<0.001$ , Figure 6.9). In contrast, the addition of human Hsp72 (10  $\mu$ g/mL) for one hour prior to incubation with increasing concentrations of PMA (0-20 ng/mL) for three hours, led to a significant decrease in the surface expression of CD36 compared to the PMA control ( $P<0.001$ , Figure 6.9). CD36 expression decreased 4-fold ( $P<0.001$ ) with Hsp72 plus 10 ng/mL PMA and almost 9-fold ( $P<0.001$ ) with Hsp72 plus 20 ng/mL PMA compared to the PMA control. At concentrations below this (1 ng/mL PMA) there was no significant difference from the PMA control (Figure 6.9). The results suggest incubation of U937 monocytes with human Hsp72 prior to treatment with PMA (10-20 ng/mL) led to a decrease in the surface expression of CD36 which was significantly different to cells treated with PMA alone ( $P<0.001$ , Figure 6.9).

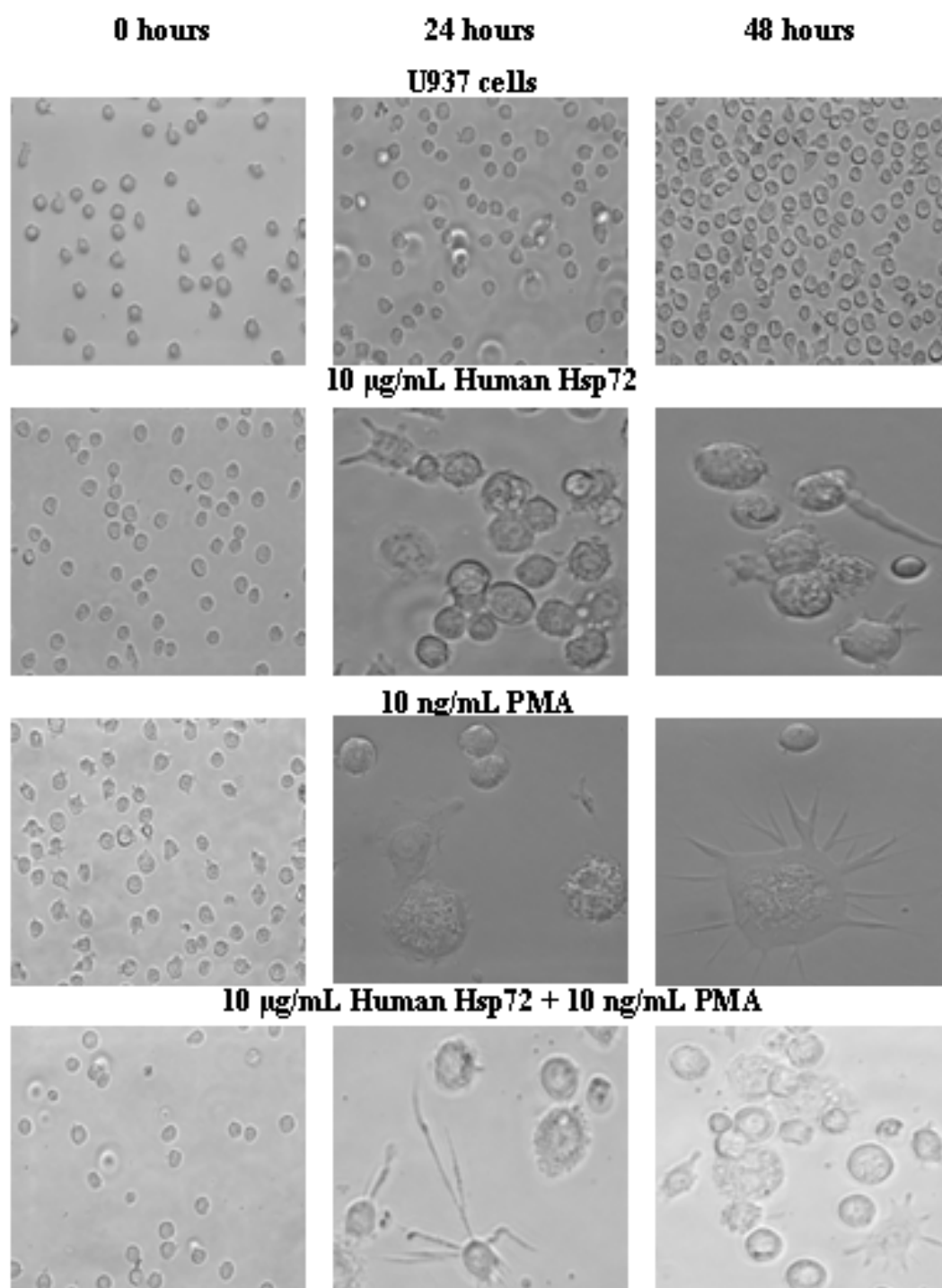


**Figure 6.9: The effect of 3 hour incubation with 1–20 ng/mL PMA at 37°C on the expression of CD36 on U937 monocytes following 1 hour incubation with 10  $\mu$ g/mL human Hsp72 at 37°C.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . \* indicates significance between mean concentrations from PMA control through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).

#### **6.3.2.6 Transformation of U937 monocytes into U937 macrophages using human Hsp72.**

The previous investigations have shown that one hour incubation with either bovine Hsp70 or human Hsp72 increased the expression of the surface markers CD36, SR-A1 and CD91, which are up-regulated during the maturation process of U937 monocytes into U937 macrophages when transformed with PMA (Figures 6.5-6.8). In order to confirm that HSPs could cause morphological changes of U937 monocytes into macrophages consistent with previous observations by several laboratories (Kulseth et al. 1998; Callahan et al. 2003; Daigneault et al. 2010), the cells were visualised using methods described in section 2.3.21 (Figure 6.10). The results suggest incubation of U937 monocytes with human Hsp72 and/or PMA for up to forty-eight hours did cause morphological changes which were different from control cells (U937 monocytes) and which were consistent with the morphology of the U937 macrophage. Untreated U937 monocytes are small, round, non-adherent cells that grow in suspension (Figure 6.10). However, after twenty-four hour incubation with either 10 ng/mL PMA or 10 µg/mL human Hsp72 alone or in combination, these cells began to form clusters, becoming larger in appearance and granular with large lysosomes, whilst some cells presented pseudopodia. The cells also became adherent to the plate surface as opposed to U937 monocytes which remained in cell suspension (Figure 6.10).



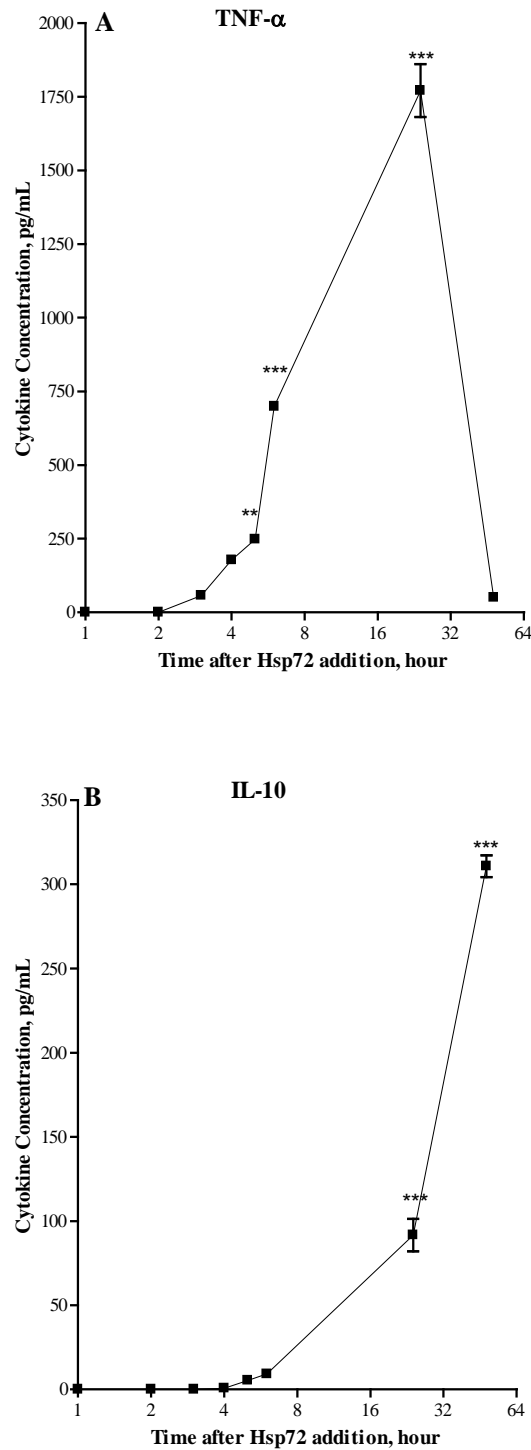
**Figure 6.10: Transformation of U937 monocytes into U937 macrophages using 1 hour incubation with 10 µg/mL human Hsp72 and/or 10 ng/mL PMA for up to 48 hours at 37°C.**

Transformation was determined through the generation of brightfield images at a magnification of x 60 on a conventional microscope and photographed on a Hamamatsu CA4742-95 camera. Data are representative of three independent experiments.

### **6.3.3 Cytokine secretion from U937 monocytes and macrophages following a forty-eight hour incubation with human Hsp72.**

Cytokine secretion from U937 monocytes and U937 macrophages following treatment with human Hsp72 (10  $\mu\text{g/mL}$ ) is presented in Figure 6.11.

Following incubation of U937 monocytes with 10  $\mu\text{g/mL}$  human Hsp72 for up to forty-eight hours, a significant increase in TNF- $\alpha$  secretion was observed compared to control cells ( $P < 0.01$  to  $P < 0.001$ , Figure 6.11A). An almost 6-fold increase ( $P < 0.01$ ) was seen after a five hour incubation with Hsp72 compared to control (Figure 6.11A) however, at incubation times below this (one-four hour) there was no significant difference from control (Figure 6.11A). TNF- $\alpha$  secretion increased almost 11-fold ( $P < 0.001$ ) when incubated for six hours with human Hsp72 and increased more than 18-fold ( $P < 0.001$ ) when incubated for twenty-four hours (Figure 6.11A). However, after forty-eight hour incubation with 10  $\mu\text{g/mL}$  human Hsp72, TNF- $\alpha$  secretion decreased almost 38-fold to levels which were not significantly different from control (Figure 6.11A). In addition, IL-10 secretion increased almost 7-fold ( $P < 0.001$ ) when U937 monocytes were incubated with 10  $\mu\text{g/mL}$  human Hsp72 for twenty-four hours compared with control cells (Figure 6.11B). At incubation times below this (one–six hour) there was no significant difference from control (Figure 6.11A). In contrast to TNF- $\alpha$  secretion, IL-10 secretion increased over 15-fold ( $P < 0.001$ ) when incubated for forty-eight hours with human Hsp72 compared to control cells (Figure 6.11B), suggesting the secretion of TNF- $\alpha$  may be suppressed by the secretion of IL-10.



**Figure 6.11: Cytokine secretion from U937 monocytes and macrophages following incubation with human Hsp72 (10  $\mu$ g/mL) for up to 48 hours at 37°C.**

(A) TNF- $\alpha$  and (B) IL-10 secretion. Data are presented as mean  $\pm$  SEM, n=3. \* indicates significant difference between mean time points from time 0 control through use of one- way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* (P<0.01), \*\*\* (P<0.001).



### **6.3.4 Treatment of U937 monocytes with a one hour heat shock at 42°C or 46°C and/or PMA.**

The previous experiments established that the administration of extracellular human Hsp72 and bovine Hsp70 (10 µg/mL) to U937 monocytes for up to forty-eight hours at 37°C promotes the differentiation of U937 monocytes to U937 macrophages. The following experiments aim to investigate whether increasing intracellular Hsp72 concentration also effects macrophage differentiation, through a one hour heat shock treatment (42°C or 46°C) and incubation with PMA. It also aims to examine whether increasing intracellular Hsp72 concentration alone could influence macrophage differentiation.

Surface expression of CD36, SR-A1 and CD91 on U937 monocytes in response to an increased intracellular Hsp72 concentration and/or PMA are presented in Figures 6.12-6.14.

#### **6.3.4.1 Surface expression of CD36 on U937 monocytes and macrophages following a one hour heat shock treatment and/or PMA.**

When U937 monocytes were stressed by heat at 42°C or 46°C for one hour, an increase in the surface expression of CD36 was observed compared to control (Figure 6.12A-B). An almost 2-fold increase was seen at 42°C and an almost 3-fold increase at 46°C compared to control cells (Figure 6.12A-B), however despite the difference in fold increase there was no significant difference from control. Following the one hour heat shock treatment at 42°C, 15 % of U937 monocytes were apoptotic, 10 % were necrotic and 75 % remained viable (Table 6.1). Furthermore, after the one hour heat shock treatment at 46°C, 20 % were apoptotic, 38 % were necrotic and 41 % remained viable (Table 6.1). Treatment of U937 monocytes with PMA also led to a significant increase in the surface expression of CD36 at three (5-fold,  $P<0.001$ ), twenty-four (10-fold,  $P<0.001$ ) and forty-eight (12-fold,  $P<0.001$ ) hours compared to control cells (Figures 6.12A-B), which are consistent with previous findings (Figure 6.2C).

In contrast to heat shock treatment alone, the addition of 10 ng/mL PMA for three hours following a one hour heat shock at 42°C or 46°C led to a significant increase in the surface expression of CD36 compared to control ( $P<0.001$ , Figures 6.12A-B) and to the PMA control ( $P<0.01$  and  $P<0.001$  respectively,

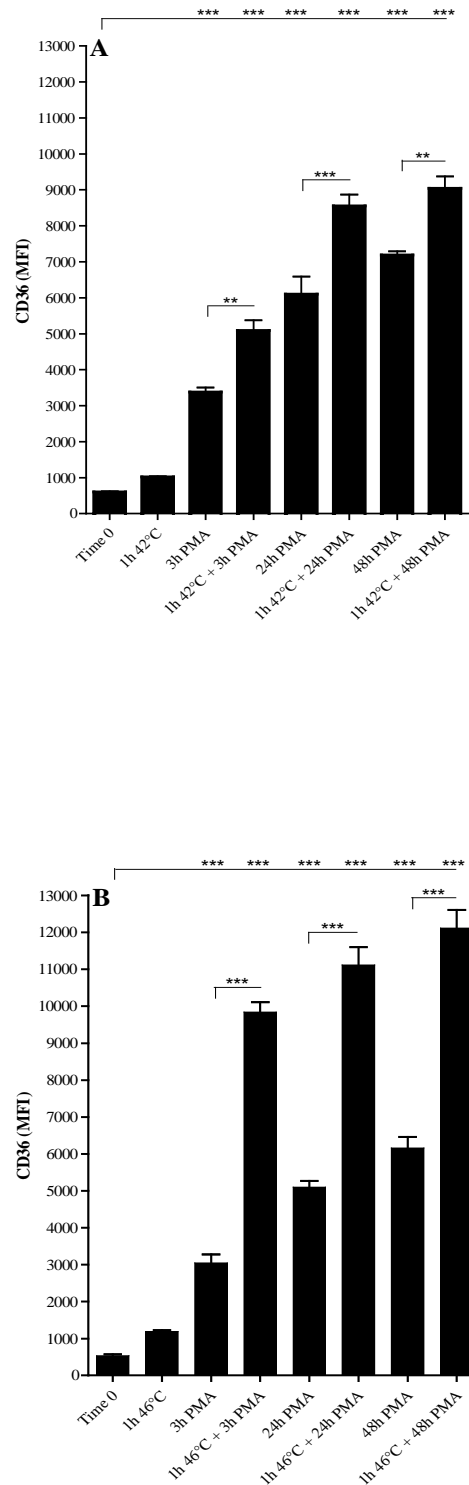
Figures 6.10A-B). These results are in contrast to those obtained in figures 6.5-6.9 using extracellularly administered HSPs where the expression of CD36 significantly decreased. A one hour 42°C heat shock plus PMA led to a significant 5-fold increase ( $P<0.001$ ) of CD36 surface expression compared to control and a 2-fold increase ( $P<0.01$ ) compared to the PMA control (Figure 6.12A). Whilst a 46°C heat shock plus PMA caused a significant 16-fold increase ( $P<0.001$ ) compared to control and an almost 3-fold increase ( $P<0.001$ ) compared to the PMA control (Figure 6.12B). The results revealed the surface expression of CD36 to increase as the length of exposure to PMA increased, 12-fold ( $P<0.001$ ) compared to control and 2-fold ( $P<0.001$ ) compared to the PMA control after twenty-four hours, and 15-fold ( $P<0.001$ ) compared to control and 2-fold ( $P<0.01$ ) compared to the PMA control after forty-eight hours following a 42°C heat shock (Figure 6.12A). Furthermore, a 20-fold ( $P<0.001$ ) increase of CD36 surface expression was also observed after twenty-four hours compared to control and a 2-fold ( $P<0.001$ ) increase compared to the PMA control following a 46°C heat shock, whilst a 23-fold ( $P<0.001$ ) increase of CD36 surface expression was observed compared to control and 2-fold ( $P<0.001$ ) increase compared to the PMA control after forty-eight hours following a 46°C heat shock (Figure 6.12B). These results suggest a one hour 46°C heat shock plus incubation with 10 ng/mL PMA to be more effective than a one hour 42°C heat shock plus incubation with 10 ng/mL PMA at increasing the surface expression of CD36 on the remaining viable population ( $P<0.001$ , Figures 6.12A-B).

**Table 6.1: The percentage of viable, apoptotic and necrotic U937 monocytes following a one hour heat shock treatment at 42°C or 46°C.**

U937 monocytes were heat shocked for one hour at 42°C or 46°C. The percentage of viable, apoptotic and necrotic U937 monocytes were then quantitatively determined using a microplate colorimetric assay (cell viability), and microplate based assays measuring caspase-3 and PI on a fluorescence microplate reader (apoptosis and necrosis).

<b>Sample</b>	<b>Cell Viability (MTS, %)</b>	<b>Apoptosis (Caspase-3, %)</b>	<b>Necrosis (PI, %)</b>
<b>Control</b>	96.7 ± 1.2	2.0 ± 0.1	1.3 ± 0.2
<b>Treated (42°C)</b>	75 ± 0.7	15.2 ± 0.3	9.8 ± 0.7
<b>Treated (46°C)</b>	41.8 ± 4.0	20.0 ± 0.2	38.2 ± 1.1
<b>Control: Apoptosis</b>	3.3 ± 0.1	95.7 ± 2.1	1 ± 0.0
<b>Control: Necrosis</b>	3.1 ± 0.6	1.9 ± 0.6	95 ± 2.5

Note: Data are presented as SEM, n=3. Control = 37°C. Caspase-3 positive control = 3 hour heat shock at 40.8°C. PI and cell viability positive control = 6 hour heat shock at 46.2°C. Seeding density  $5 \times 10^5$ .



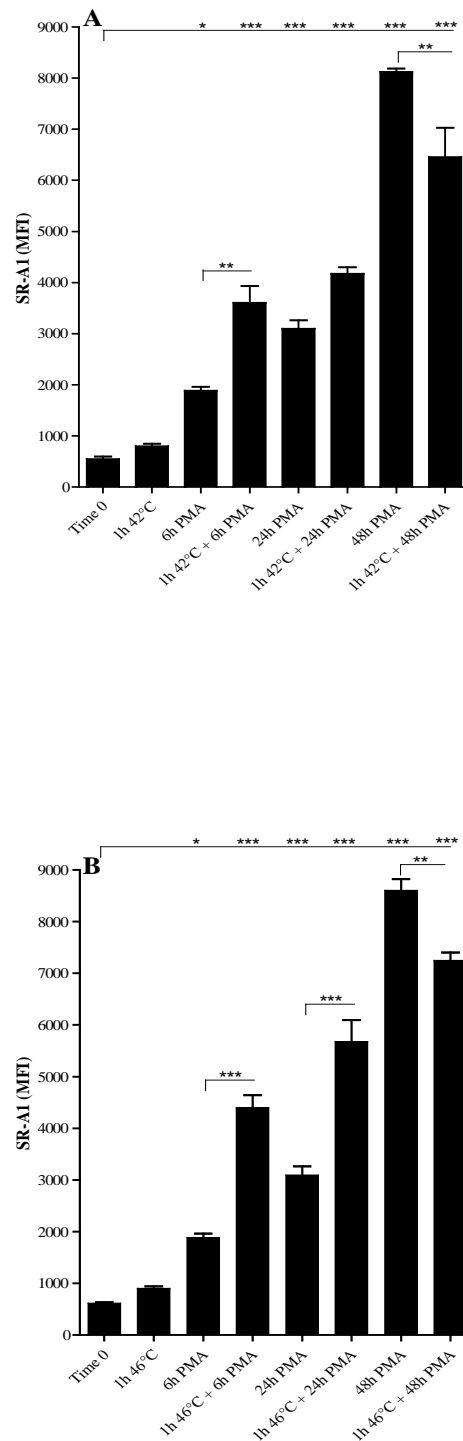
**Figure 6.12: The effect of 1 hour 42°C (A) or 46°C (B) heat shock treatment on PMA induced expression of CD36 on U937 monocytes and macrophages.** Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean treatments from time 0 control or the relevant PMA control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* (P<0.01), \*\*\* (P<0.001).

#### **6.3.4.2 Surface expression of SR-A1 on U937 monocytes and macrophages following a one hour heat shock treatment and/or PMA.**

Following incubation of U937 monocytes for one hour at 42°C or 46°C, the surface expression of SR-A1 increased compared to control cells (Figure 6.13A-B). A 1-fold increase was seen at 42°C and an almost 2-fold increase at 46°C compared to control (Figure 6.13A-B), however there was no significant difference from control cells. Treatment of U937 monocytes with PMA also led to a significant increase in the surface expression of SR-A1 at six (4-fold,  $P<0.05$ ), twenty-four (6-fold,  $P<0.001$ ) and forty-eight (18-fold,  $P<0.001$ ) hours compared to control cells (Figure 6.13A-B), which are consistent with previous findings (Figure 6.3C).

In comparison to heat shock treatment alone, the addition of 10 ng/mL PMA for six hours following a one hour heat shock at 42°C or 46°C led to a significant increase in the surface expression of SR-A1 compared to control ( $P<0.001$ , Figure 6.13A-B) and to the PMA control ( $P<0.01$ , Figure 6.11A,  $P<0.001$ , Figure 6.13B). SR-A1 expression significantly increased 6-fold ( $P<0.001$ ) compared to control and 2-fold ( $P<0.01$ ) compared to the PMA control after a one hour 42°C heat shock plus PMA (Figure 6.13A). Similarly, an almost 8-fold ( $P<0.001$ ) increase of SR-A1 expression was observed compared to control and a 3-fold increase ( $P<0.001$ ) compared to the PMA control after a one hour 46°C heat shock plus PMA (Figure 6.13B). Similar to the expression of CD36, the results observed the surface expression of SR-A1 to increase as the exposure time with PMA increased, almost 9-fold ( $P<0.001$ ) after twenty-four hours compared to control, however compared to the PMA control the results were found to be not significant (Figure 6.13A). A 13-fold ( $P<0.001$ ) increase was also observed compared to control and 1-fold ( $P<0.001$ ) increase compared to the PMA control after forty-eight hours following a 42°C heat shock (Figure 6.13A). Furthermore, SR-A1 expression significantly increased 11-fold ( $P<0.001$ ) compared to control and 2-fold ( $P<0.001$ ) compared to the PMA control after twenty-four hours (Figure 6.13B), and 14-fold ( $P<0.001$ ) compared to control and 1-fold ( $P<0.001$ ) compared to the PMA control after forty-eight hours following a 46°C heat shock (Figure 6.13B). These results suggest a one hour 46°C heat shock plus incubation with 10 ng/mL PMA to be more effective than a one hour 42°C heat shock plus

incubation with 10 ng/mL PMA at increasing the surface expression of SR-A1 on the remaining viable population ( $P < 0.001$ , Figure 6.13A-B).



**Figure 6.13: The effect of 1 hour 42°C (A) or 46°C (B) heat shock treatment on PMA induced expression of SR-A1 on U937 monocytes and macrophages.**

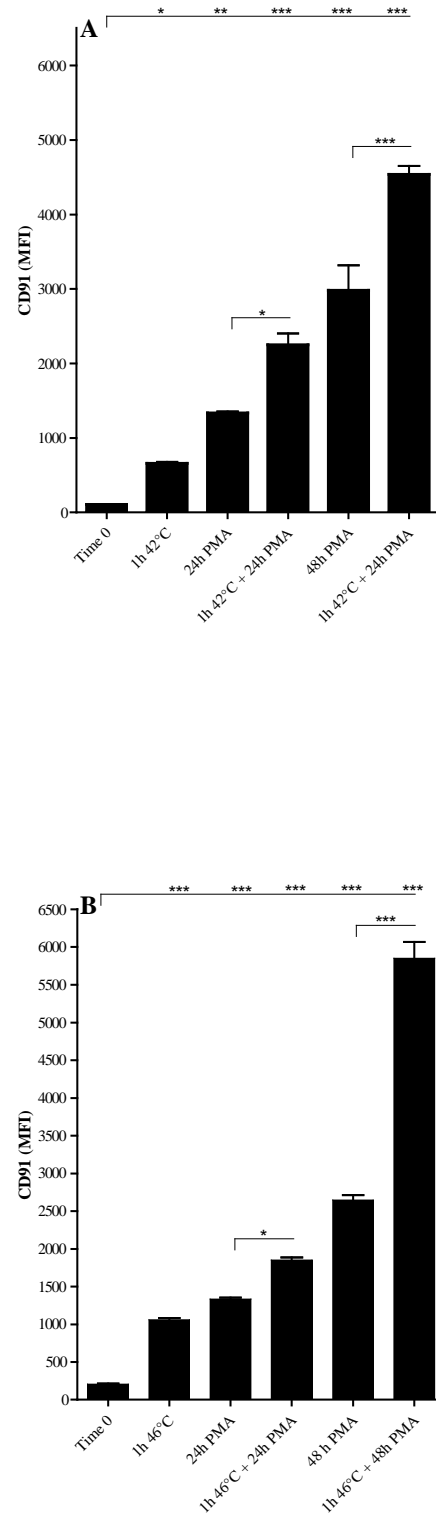
Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean treatments from time 0 control or the relevant PMA control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001).

#### **6.3.4.3 Surface expression of CD91 on U937 monocytes and macrophages following a one hour heat shock treatment and/or PMA.**

In contrast to the surface expression of both CD36 and SR-A1, the incubation of U937 monocytes for one hour at 42°C or 46°C led to a significant increase in the surface expression of CD91 compared to control cells ( $P<0.05$  and  $P<0.001$  respectively, Figure 6.14A-B). An almost 4-fold increase ( $P<0.05$ , Figure 6.14A) was seen at 42°C and a 5-fold increase ( $P<0.001$ , Figure 6.14B) at 46°C compared to control (Figure 6.14A-B). In addition, treatment of U937 monocytes with PMA also led to a significant increase in the surface expression of CD91 after twenty-four (7-fold,  $P<0.01$  to  $P<0.001$ ) and forty-eight (13-fold increase,  $P<0.001$ ) hours compared to control cells (Figure 6.14A-B), which are consistent with previous findings (Figure 6.4C).

The addition of 10 ng/mL PMA for twenty-four hours following a one hour heat shock at 42°C or 46°C, also led to a significant increase in the surface expression of CD91 compared to control ( $P<0.001$ , Figure 6.14A and  $P<0.001$ , Figure 6.14B) and to the PMA control ( $P<0.05$ , Figure 6.14A and  $P<0.05$ , Figure 6.14B). CD91 expression increased 8-fold ( $P<0.001$ ) compared to control and 1-fold ( $P<0.05$ ) compared to the PMA control after a 42°C heat shock plus PMA for twenty-four hours (Figure 6.14A). Similarly, an almost 9-fold increase ( $P<0.001$ ) of CD91 surface expression was also seen after a one hour 46°C heat shock plus PMA for twenty-four hours compared to control and a 2-fold increase ( $P<0.05$ ) compared to the PMA control (Figure 6.14B). Furthermore, as the exposure of U937 monocytes to PMA increased to forty-eight hours, an almost 24-fold ( $P<0.001$ ) increase of CD91 expression was observed compared to control and a 2-fold increase ( $P<0.001$ ) compared to the PMA control after a 42°C heat shock plus PMA (Figure 6.14A). In addition, a 26-fold ( $P<0.001$ ) increase was observed compared to control and a 2-fold increase ( $P<0.001$ ) of CD91 surface expression compared to the PMA control was observed after a 46°C heat shock plus PMA after forty-eight hours (Figure 6.14B). The results suggest a one hour 46°C heat shock plus incubation with 10 ng/mL PMA to be more effective than a one hour 42°C heat shock plus incubation with 10 ng/mL PMA at increasing the surface expression of CD91 on the remaining viable cells ( $P<0.001$ , Figure 6.14A-B).

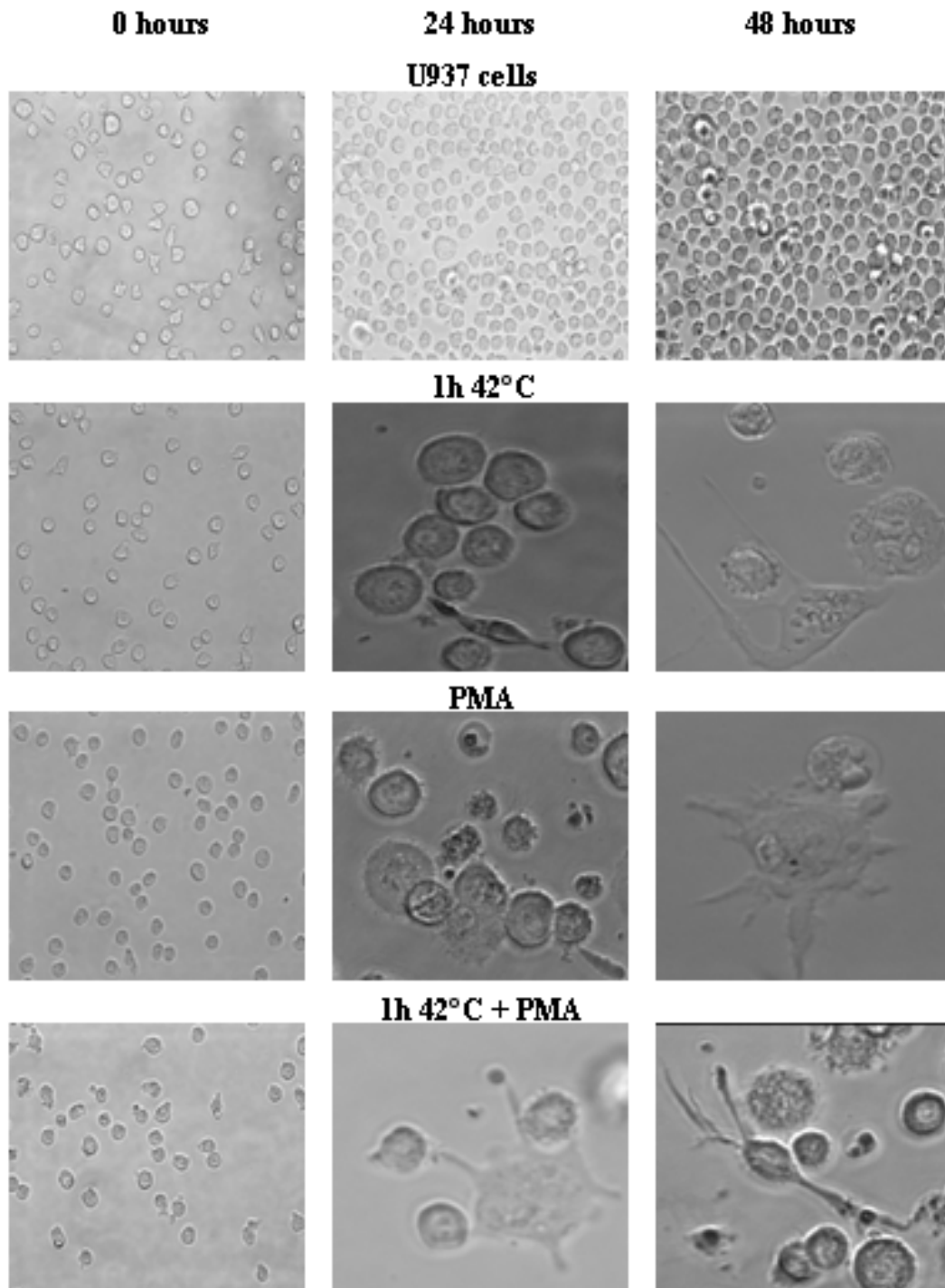




**Figure 6.14: The effect of 1 hour 42°C (A) or 46°C (B) heat shock treatment on PMA induced expression of CD91 on U937 monocytes and macrophages.** Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean treatments from time 0 control or the relevant PMA control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001).

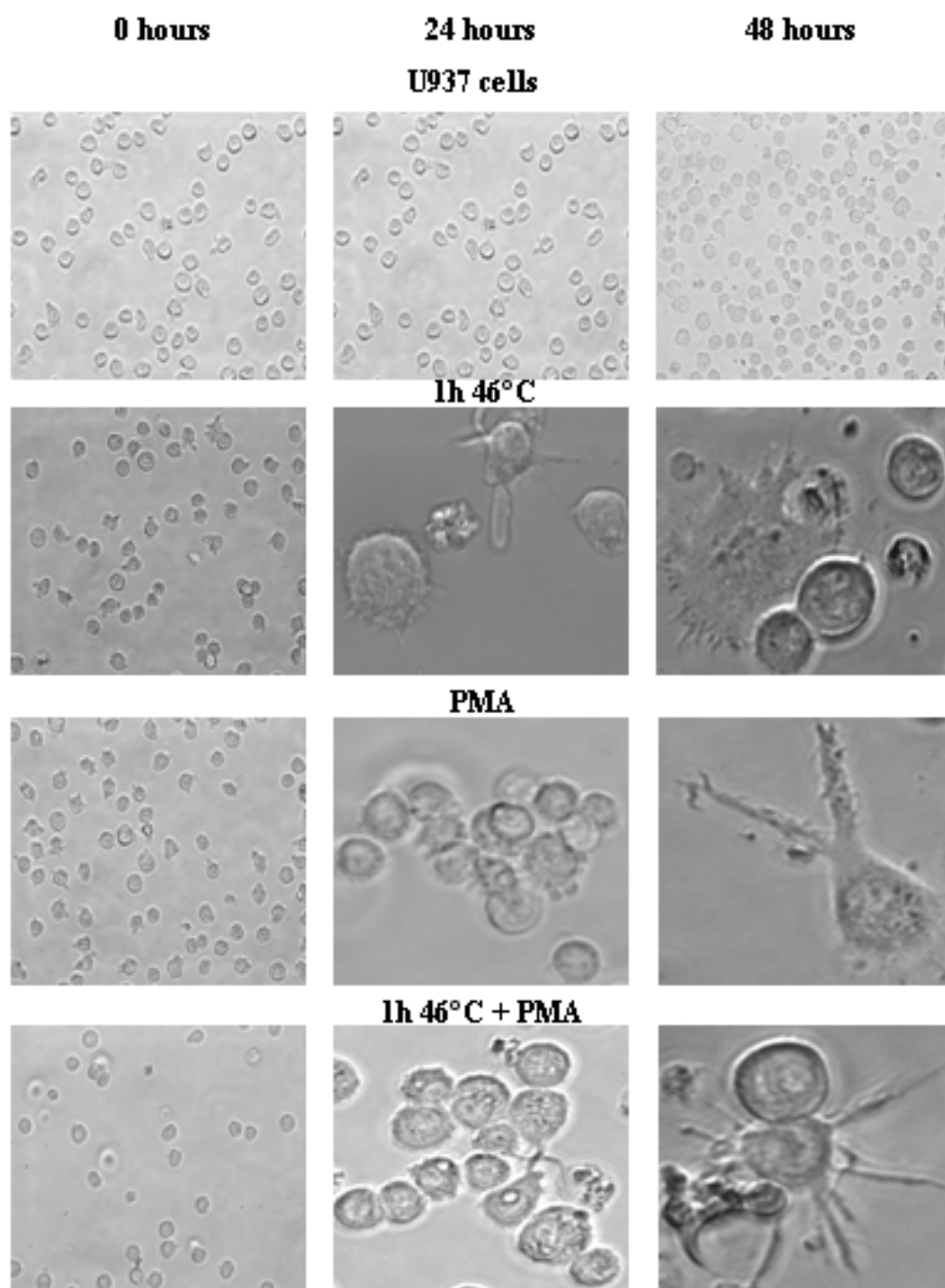
#### **6.3.4.4 Transformation of U937 monocytes into U937 macrophages using a heat treatment protocol.**

The results of the heat treatment protocol suggest a one hour heat shock at either 42°C or 46°C alone or in combination with PMA increase the expression of the surface markers CD36, SR-A1 and CD91 (Figures 6.12-6.14). In order to confirm that heat shock and PMA did cause morphological changes consistent with the previous observations seen in figure 6.10, the cells were visualised using methods described in section 2.3.21 (Figures 6.15-16). The results suggest incubation of U937 monocytes at elevated temperatures alone or with PMA did cause morphological changes which were different from control U937 monocytes. Following twenty-four hours after treatment with heat shock or PMA, the cells became adherent to the plate surface compared to U937 monocytes which remained in cell suspension (Figures 6.15-6.16). From twenty-four hours to forty-eight hours after incubation, the cells became larger and granular in appearance and began to form clusters (Figures 6.15-6.16) and some cells presented pseudopodia (Figures 6.15-6.16).



**Figure 6.15: Transformation of U937 monocytes into U937 macrophages using 1 hour heat shock treatment at 42°C and/or 10 ng/mL PMA for up to 48 hours at 37°C.**

Transformation was determined through the generation of brightfield images at a magnification of x 60 on a conventional microscope and photographed on a Hamamatsu CA4742-95 camera. Data are representative of three independent experiments.



**Figure 6.16: Transformation of U937 monocytes into U937 macrophages using 1 hour heat shock treatment at 46°C and/or 10 ng/mL PMA for up to 48 hours at 37°C.**

Transformation was determined through the generation of brightfield images at a magnification of x 60 on a conventional microscope and photographed on a Hamamatsu CA4742-95 camera. Data are representative of three independent experiments.

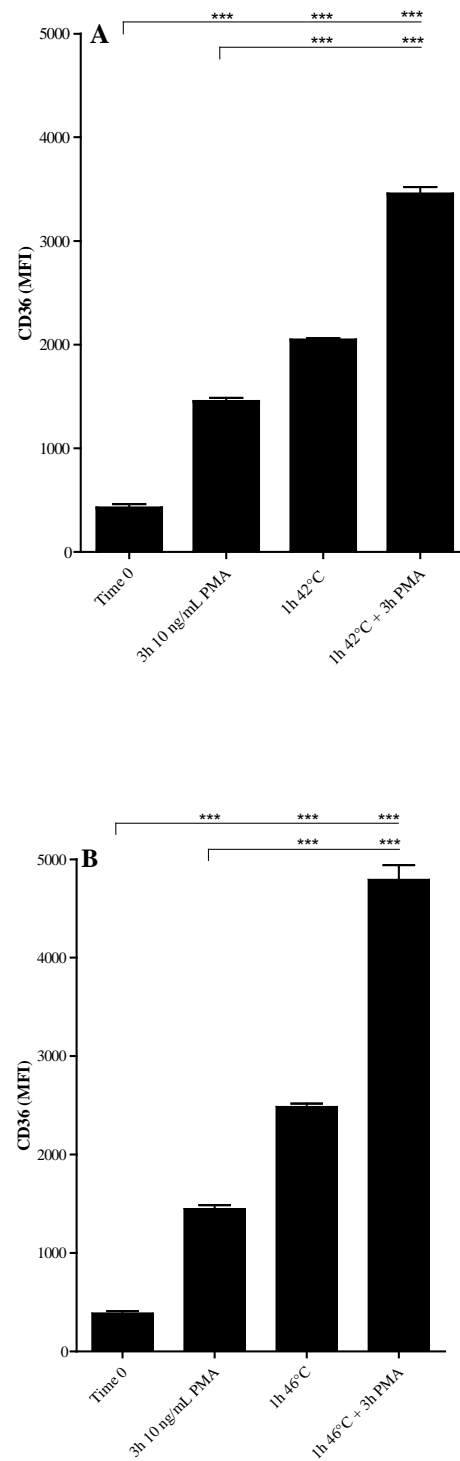
### **6.3.5 Treatment of human monocytes with a one hour heat shock at 42°C or 46°C and/or PMA.**

The previous experiments determined that increasing intracellular Hsp72 concentration (as demonstrated in chapter 3), through a one hour heat shock treatment at 42°C or 46°C and incubation with PMA for up to forty-eight hours at 37°C promotes the differentiation of U937 monocytes to U937 macrophage. The main objective of the following part of this study was to investigate the influence of a one hour heat shock treatment (42°C or 46°C) alone or in combination with PMA on the expression of CD36 on human monocytes (Figure 6.17A-B).

When human monocytes were stressed by elevated temperatures of 42°C or 46°C for one hour, an increase in the surface expression of CD36 was observed compared to control (Figure 6.17A-B). An almost 5-fold increase ( $P<0.001$ ) was seen at 42°C (Figure 6.17A) and an almost 7-fold increase ( $P<0.05$ ) at 46°C compared to control cells (Figure 6.17B). In addition, treatment of human monocytes with PMA for three hours also led to a significant, 4-fold increase ( $P<0.001$ ) in the surface expression of CD36 compared to control cells (Figure 6.17A-B), which are similar to the findings of cultured U937 monocytes (Figure 6.2C).

In contrast to heat shock treatment alone, the addition of 10 ng/mL PMA for three hours following a one hour heat shock at 42°C or 46°C led to a further significant increase in the surface expression of CD36 compared to control ( $P<0.001$ , Figure 6.15A-B) and to the PMA control ( $P<0.001$ , Figures 6.17A-B). These results are consistent with the results obtained in figures 6.12-6.14 using U937 monocytes and U937 macrophages. A one hour 42°C heat shock plus PMA led to an 8-fold increase ( $P<0.001$ ) of CD36 surface expression compared to control and an almost 2-fold increase ( $P<0.001$ ) compared to the PMA control (Figure 6.17A). Whilst a 46°C heat shock plus PMA led to an almost 13-fold increase ( $P<0.001$ ) compared to control and a 2-fold increase ( $P<0.001$ ) compared to the PMA control (Figure 6.17B). These results, similar to those observed with U937 monocytes (Figures 6.12-6.14), suggest a one hour 46°C heat shock plus incubation with 10 ng/mL PMA for three hours to be more

effective than a one hour 42°C heat shock plus incubation with 10 ng/mL PMA at increasing the surface expression of CD36 ( $P < 0.001$ , Figure 6.17A-B).



**Figure 6.17: The effect of 1 hour 42°C (A) or 46°C (B) heat shock treatment on PMA induced expression of CD36 on human monocytes.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean treatments from time 0 control or the PMA control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).

### 6.3.6 Treatment of U937 monocytes with supernatant enriched with Hsp72 following a 42°C or 46°C heat shock treatment.

The previous experiments established both externally applied HSPs (10 µg/mL) and increased intracellular Hsp72 concentration (demonstrated in chapter 3) promotes the differentiation of U937 monocytes to U937 macrophages. The following experiments aim to determine whether 42°C supernatant or 46°C supernatant enriched with Hsp72 will also influence macrophage differentiation.

#### 6.3.6.1 Hsp72 release from U937 monocytes following a one hour heat shock treatment at 42°C or 46°C.

In order to confirm that the heat shock protocol did induce the release of Hsp72 from U937 monocytes, the supernatant from these cells was analysed for the expression of the stress-inducible protein Hsp72 by ELISA. The results showed non-detectable levels of Hsp72 in the supernatant from U937 monocytes incubated at 37°C for one hour. In contrast, an 86-fold increase in Hsp72 release was observed in the supernatant from U937 monocytes heat shocked at 42°C compared to the 37°C control (Table 6.2). Furthermore, an almost 200-fold increase in Hsp72 release was found in the supernatant from U937 monocytes heat shocked at 46°C compared to the 37°C control (Table 6.2). These results suggest the heat shock protocol was sufficient to induce Hsp72 release from U937 monocytes.

**Table 6.2: Hsp72 release from 42°C or 46°C heat shocked U937 monocytes.**

U937 monocytes were heat shocked for one hour at 42°C (75 % viable, 15 % apoptotic, 10 % necrotic) or 46°C (42 % viable, 20 % apoptotic, 38 % necrotic). Samples were then centrifuged (400 g, 5 minutes, RT) and the supernatant was analysed for Hsp72 by ELISA.

Sample	Hsp72 (ng/mL)
Control (37°C)	0.0 ± 0.0
Treated (42°C)	86.2 ± 0.5
Treated (46°C)	189.5 ± 2.5

Note: Data are presented as SEM, n=3. Control = 37°C. Seeding density 5 x 10<sup>5</sup>.



#### **6.3.6.2 Surface expression of CD36 on U937 monocytes following incubation with supernatant enriched with Hsp72 released from heat shocked U937 monocytes.**

Surface expression of CD36 on U937 monocytes in response to three hour treatment with supernatant enriched with Hsp72 from 42°C or 46°C heat shocked U937 monocytes is presented in Figures 6.18-6.20.

Following incubation of U937 monocytes with supernatant containing Hsp72 released into 42°C or 46°C supernatant led to a significant increase in the surface expression of CD36 compared to control ( $P<0.001$ , Figure 6.18) and to the 37°C control ( $P<0.001$ , Figure 6.18). An almost 5-fold increase ( $P<0.001$ ) was seen with Hsp72 released into 42°C supernatant compared to control and to the 37°C control, and a 12-fold increase ( $P<0.001$ ) with Hsp72 released into 46°C supernatant compared to control and to the 37°C control (Figure 6.18). Treatment of cells with supernatant from U937 monocytes incubated at 37°C for one hour had no effect on CD36 expression (Figure 6.18). The results suggest Hsp72 released into 46°C supernatant to be more effective than Hsp72 released into 42°C supernatant at increasing the surface expression of CD36 on U937 monocytes ( $P<0.001$ , Figure 6.18).

#### **6.3.6.3 Surface expression of CD36 on U937 monocytes following incubation with supernatant enriched with Hsp72 and/or anti-human Hsp72.**

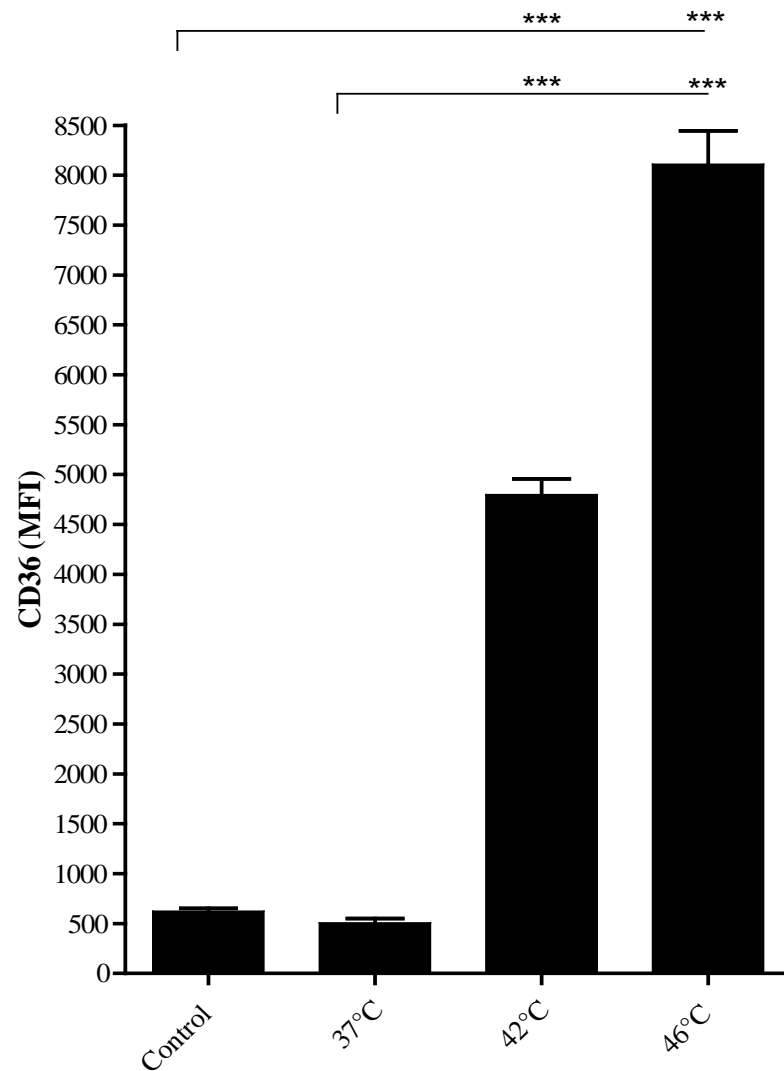
Consistent with previous findings (Figure 6.18), treatment of U937 monocytes with Hsp72 released into 42°C or 46°C supernatant led to a significant increase in the surface expression of CD36. An almost 8-fold increase ( $P<0.001$ ) and a 13-fold increase ( $P<0.001$ ) was observed with supernatant enriched with Hsp72 released into 42°C (Figure 6.18A) or 46°C supernatant respectively (Figure 6.19B), compared to the 37°C control. In contrast, the addition of anti-human Hsp72 to the 42°C or 46°C Hsp72 enriched supernatants prior to incubation with U937 monocytes for three hours, led to a significant decrease in the surface expression of CD36 compared to the 42°C ( $P<0.001$ , Figure 6.19A) or 46°C samples without anti-human Hsp72 ( $P<0.05$  to  $P<0.001$ , Figure 6.19B). CD36 expression did not alter with a 1:500 or a 1:250 dilution of anti-human Hsp72 incubated with Hsp72 enriched 42°C supernatant compared to the 42°C control

(Figure 6.19A). Similarly, a 1:500 dilution of anti-human Hsp72 incubated with Hsp72 enriched 46°C supernatant did not alter CD36 surface expression compared to the 46°C control (Figure 6.19B). However, a 1-fold decrease ( $P<0.05$ ) of CD36 surface expression was observed with a 1:250 dilution of anti-human Hsp72 incubated with Hsp72 enriched 46°C supernatant compared to 46°C control (Figure 6.19B). CD36 surface expression also decreased over 2-fold ( $P<0.001$ ) in both treatments with the increased dilution of 1:100 anti-human Hsp72 plus Hsp72 enriched 42°C (Figure 6.19A) or 46°C (Figure 6.19B) supernatants compared to the 42°C and 46°C controls (Figure 6.19A-B). Furthermore, a 1:50 and a 1:10 dilution of anti-human Hsp72 led to a significant decrease in the surface expression of CD36 ( $P<0.001$ , Figure 6.19A-B). A 2-fold ( $P<0.001$ ) and 4-fold ( $P<0.001$ ) decrease was observed with Hsp72 enriched 42°C supernatant (Figure 6.19A) and an almost 5-fold ( $P<0.001$ ) and 9-fold decrease ( $P<0.001$ ) of CD36 expression observed with Hsp72 enriched 46°C supernatant (Figure 6.19B). Treatment of cells with 1:10 dilution of anti-Hsp72 alone had no effect on CD36 expression compared to the 37°C control (Figures 6.19A-B). Hsp72 enriched 46°C supernatant incubated with anti-Hsp72 was found to be more effective than Hsp72 enriched 42°C supernatant incubated with anti-Hsp72 at not increasing the surface expression of CD36 ( $P<0.001$ , Figures 6.19A-B).

#### **6.3.6.4 Surface expression of CD36 on U937 monocytes following incubation with supernatant enriched with Hsp72 and/or Brij®-98.**

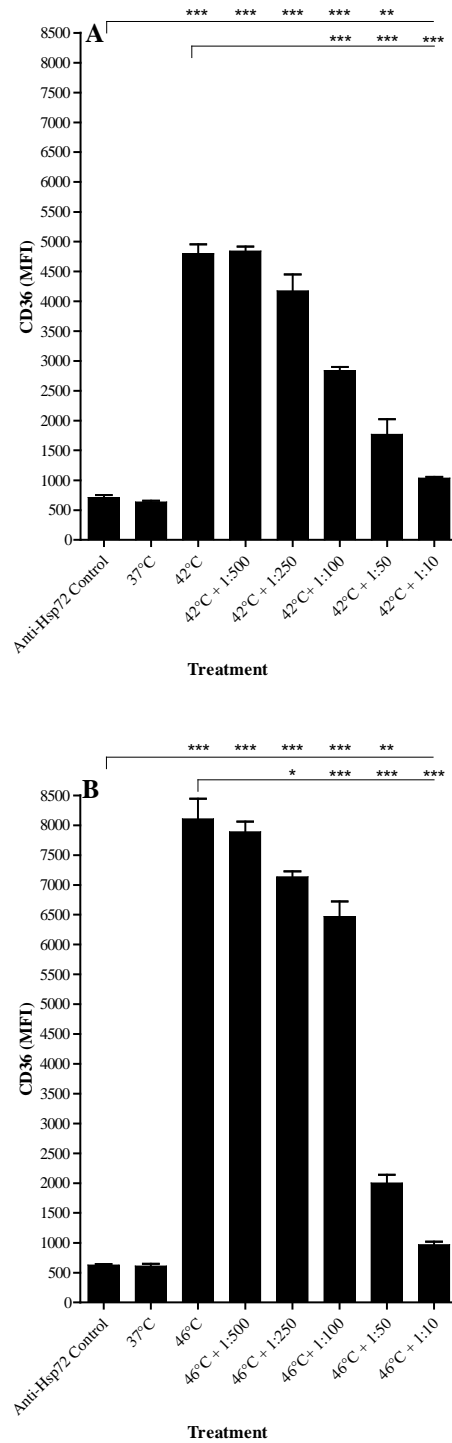
Incubation of U937 monocytes with Hsp72 enriched 42°C or 46°C supernatants caused a significant, 7-fold increase ( $P<0.001$ ) and an almost 12-fold increase ( $P<0.001$ ) in the surface expression of CD36 respectively, when compared to the 37°C control (Figure 6.20). The addition of Brij®-98 (0.5 %) to the heat shocked supernatants containing released Hsp72 for ten minutes at 4°C, prior to a three hour incubation of cells with Hsp72 enriched 42°C or 46°C supernatants led to a significant increase in the surface expression of CD36 compared to the 37°C control ( $P<0.001$ , Figure 6.20). CD36 expression increased 11-fold ( $P<0.001$ ) compared to the 37°C control and almost 2-fold ( $P<0.001$ ) compared to Hsp72 enriched 42°C supernatant, when incubated with 0.5 % Brij®-98 plus Hsp72 enriched 42°C supernatant (Figure 6.20). Similarly, a 15-fold increase ( $P<0.001$ )

of CD36 surface expression was also observed compared to the 37°C control and an almost 2-fold increase ( $P<0.001$ ) compared to Hsp72 enriched 46°C supernatant, when incubated with 0.5 % Brij®-98 plus Hsp72 enriched 46°C supernatant (Figure 6.20). Treatment of U937 monocytes with 0.5 % Brij®-98 alone had no effect on CD36 expression compared to the 37°C control (Figure 6.20). Hsp72 enriched 46°C supernatant incubated with 0.5 % Brij®-98 was found to be more effective than Hsp72 enriched 42°C supernatant incubated with Brij®-98 at increasing the surface expression of CD36 ( $P<0.001$ , Figure 6.20).



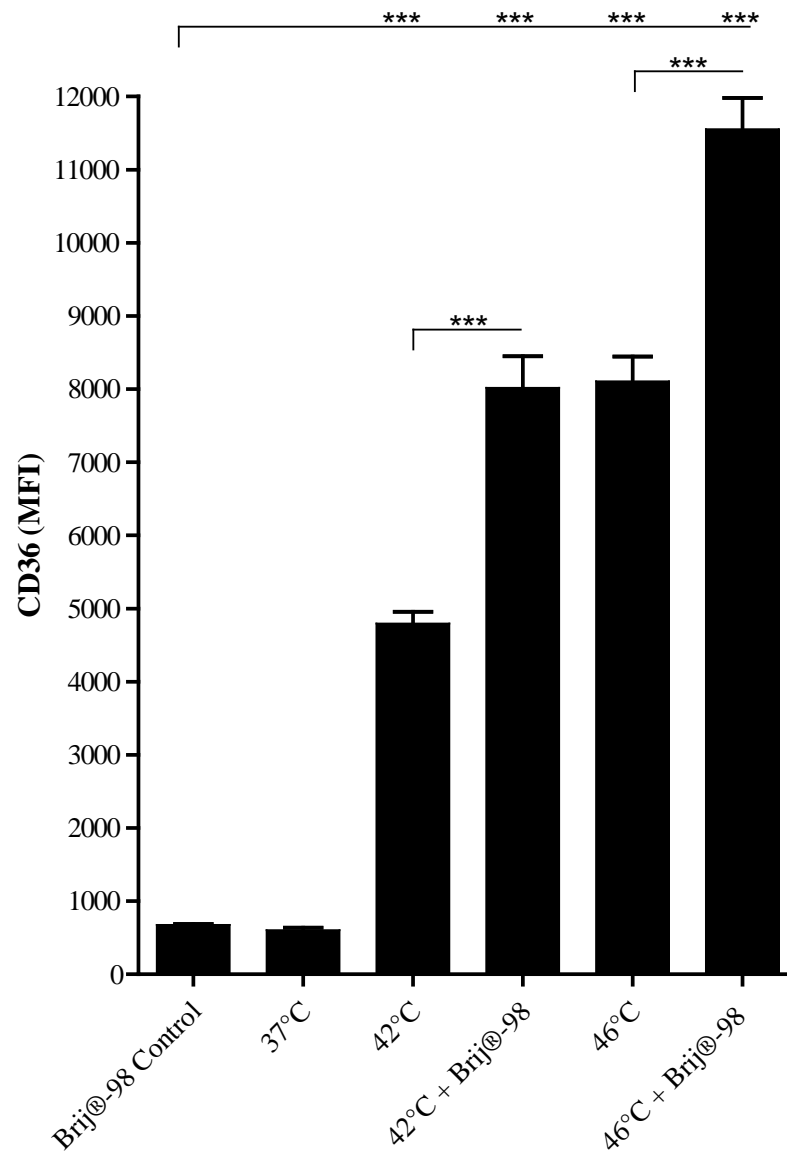
**Figure 6.18: The effect of supernatant enriched with Hsp72 following a 42°C or 46°C heat shock treatment on the induced expression of CD36 on U937 monocytes.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean temperatures from the untreated control or the 37°C control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



**Figure 6.19: The effect of supernatant enriched with Hsp72 following a 42°C (A) or 46°C (B) heat shock treatment and/or anti-human Hsp72 (1:500-1:10 dilution) on the induced expression of CD36 on U937 monocytes.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean concentrations from the 37°C control or the 42°C or 46°C supernatants without anti-human Hsp72 through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).



**Figure 6.20: The effect of supernatant enriched with Hsp72 following a 42°C or 46°C heat shock treatment and/or Brij®-98 (0.5%) on the induced expression of CD36 on U937 monocytes.**

Data are presented as mean  $\pm$  SEM, n=3. Significance shown as difference between mean treatments from the 37°C control or the 42°C or 46°C supernatants without Brij®-98 (0.5 %) through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* (P<0.01), \*\*\* (P<0.001).

### **6.3.7 Cytokine secretion from U937 monocytes following incubation with supernatant enriched with Hsp72 and/or anti-human Hsp72.**

Cytokine secretion from U937 monocytes following treatment with supernatant enriched with Hsp72 is presented in Figure 6.21.

Following incubation of U937 monocytes with Hsp72 released into 42°C or 46°C supernatant for three hours led to a significant increase in TNF- $\alpha$  secretion compared to the 37°C control ( $P < 0.001$ , Figure 6.21). An almost 3-fold increase ( $P < 0.001$ ) in TNF- $\alpha$  secretion was seen after a three hour incubation with Hsp72 enriched 42°C supernatant compared to the 37°C control (Figure 6.21), and an almost 34-fold increase ( $P < 0.001$ ) with Hsp72 enriched 46°C supernatant compared to the 37°C control (Figure 6.21). In contrast, non detectable amounts of secreted IL-10 were measured in the supernatants from U937 monocytes incubated with Hsp72 enriched 42°C or 46°C supernatant for three hours compared to the 37°C control (Figure 6.21).

The addition of 1:100 dilution of anti-human Hsp72 to the heat shocked supernatants containing released Hsp72 for one hour at 37°C, prior to a three hour incubation of U937 monocytes with Hsp72 enriched 42°C or 46°C supernatant, led to a significant decrease in the secretion of TNF- $\alpha$  from U937 monocytes, to levels which were not significantly different from the 37°C control (Figure 6.21). In addition, treatment of U937 monocytes with a 1:100 dilution of anti-human Hsp72 alone did not activate TNF- $\alpha$  or IL-10 secretion compared to the 37°C control (Figure 6.21).

**Table 6.3: Cytokine secretion from U937 monocytes following incubation with supernatant enriched with Hsp72 and/or anti-human Hsp72 (1:100 dilution) for 3 hours at 37°C.**

U937 monocytes were heated at 42°C or 46°C for 1 hour. Samples were then centrifuged (400 g, 5 minutes, 25°C) and the supernatant was removed and incubated with 1:100 anti-human Hsp72 for 1 hour before being applied to U937 monocytes for 3 hours. Significance shown as difference between mean treatments from the 37°C control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* (P<0.001).

Sample	TNF- $\alpha$ Concentration (pg/mL)	IL-10 Concentration (pg/mL)
<b>Control: Anti-Hsp72</b>	0 $\pm$ 0.0	0 $\pm$ 0.0
<b>Control: 37°C</b>	0 $\pm$ 0.0	0 $\pm$ 0.0
<b>42°C Supernatant</b>	2.8 $\pm$ 1.3***	0 $\pm$ 0.0
<b>42°C, anti-Hsp72</b>	0 $\pm$ 0.0	0 $\pm$ 0.0
<b>46°C Supernatant</b>	34.9 $\pm$ 1.9***	0 $\pm$ 0.0
<b>46°C, anti-Hsp72</b>	0 $\pm$ 0.0	0 $\pm$ 0.0

Note: Data are presented as mean  $\pm$  SEM, n=3. Control = 1:100 dilution anti-human Hsp72 or 37°C. Seeding density 5 x 10<sup>5</sup>.



### **6.3.8 Treatment of U937 monocytes with heat shocked, 42°C or 46°C U937 monocytes.**

The previous experiment determined that Hsp72 enriched supernatant from heat shocked monocytes influenced the differentiation of U937 monocytes to U937 macrophages. The following experiments aim to investigate whether heat shocked 42°C U937 monocytes or heat shocked 46°C U937 monocytes also effect macrophage differentiation.

Surface expression of CD36 on U937 monocytes in response to three hour incubation with 42°C or 46°C heat shocked U937 monocytes are presented in Figures 6.21-6.22.

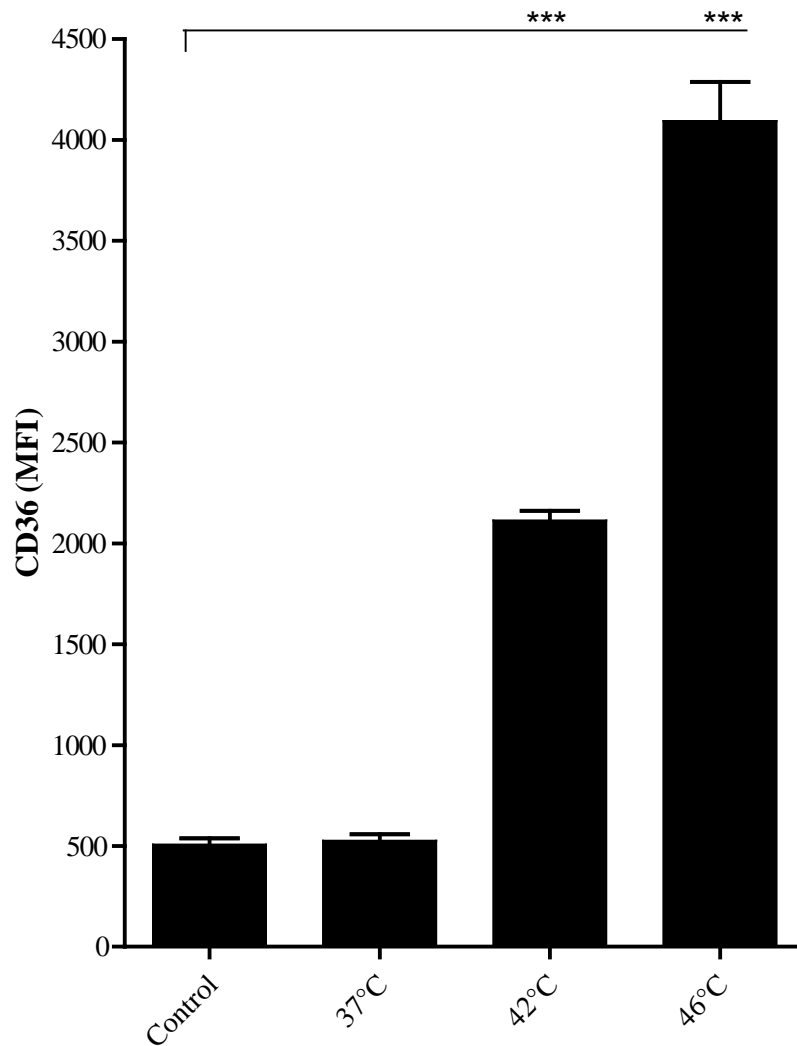
#### **6.3.8.1 Surface expression of CD36 on U937 monocytes following incubation with heat shocked U937 monocytes.**

Incubation of U937 monocytes with 42°C or 46°C heat shocked monocytes led to a significant increase in the surface expression of CD36 compared to control cells ( $P<0.001$ , Figure 6.21). An almost 4-fold increase ( $P<0.001$ ) was seen with 42°C heat shocked U937 monocytes and an 8-fold increase ( $P<0.001$ ) with 46°C heat shocked U937 monocytes compared to control cells (Figure 6.21). Treatment of cells with U937 monocytes incubated at 37°C for one hour had no effect on CD36 expression (Figure 6.21). Heat shocked 46°C U937 monocytes were found to be more effective than heat shocked 42°C monocytes at increasing the surface expression of CD36 on U937 monocytes ( $P<0.001$ , Figure 6.21).

#### **6.3.8.2 Surface expression of CD36 on U937 monocytes following incubation with heat shocked U937 monocytes and/or anti-human Hsp72.**

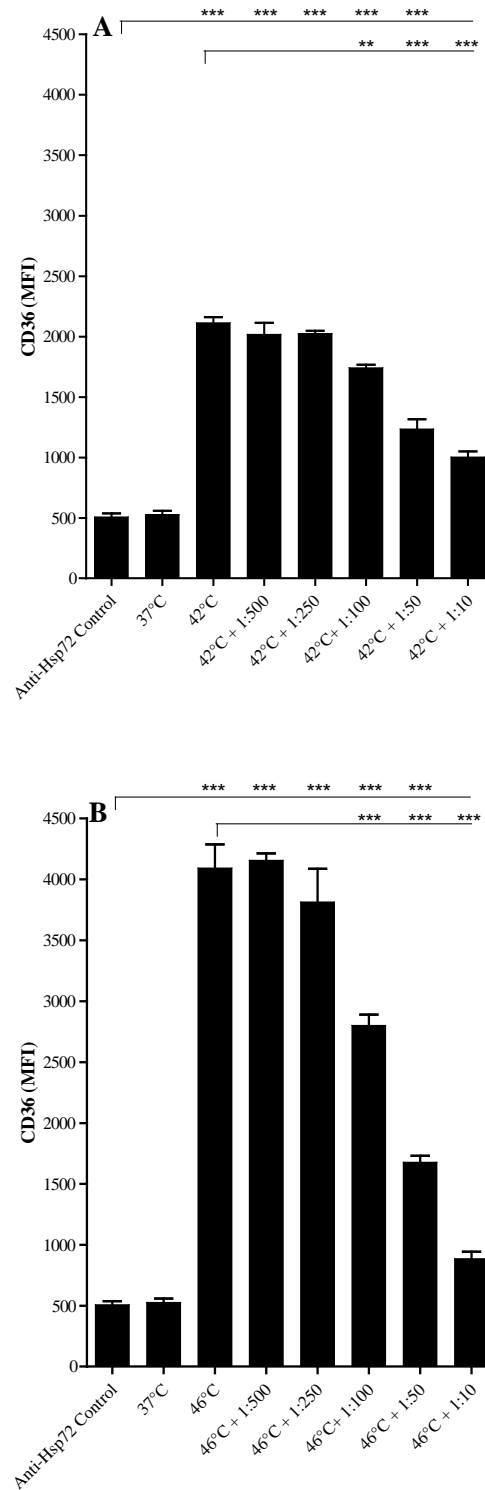
Consistent with the findings shown in figure 6.21, treatment of U937 monocytes with 42°C or 46°C heat shocked monocytes led to a significant increase in the surface expression of CD36. An almost 4-fold ( $P<0.001$ ) increase and a 9-fold increase ( $P<0.001$ ) was observed with 42°C heat shocked monocytes (Figure 6.22A) and 46°C heat shocked monocytes respectively (Figure 6.22B) compared to the 37°C control (Figure 6.22A-B). In contrast, the addition of anti-human Hsp72 to the 42°C or 46°C heat shocked monocytes for one hour at 37°C, prior to incubation with U937 monocytes for three hours led to a significant decrease

in the surface expression of CD36 compared to the 42°C ( $P<0.01$  to  $P<0.001$ , Figure 6.22A) or 46°C samples without anti-human Hsp72 ( $P<0.001$ , Figure 6.22B). CD36 expression did not significantly alter with a 1:500 or a 1:250 dilution of anti-human Hsp72 incubated with 42°C or 46°C heat shocked monocytes compared to the 42°C or 46°C controls (Figure 6.22A). However, CD36 surface expression did decrease over 2-fold ( $P<0.01$  and  $P<0.001$ ) in both treatments when incubated with a 1:100 dilution of anti-human Hsp72 plus 42°C heat shocked monocytes ( $P<0.01$ , Figure 6.22A), and 46°C heat shocked monocytes ( $P<0.001$ , Figure 6.22B) compared to the 42°C or 46°C controls (Figure 6.22A-B). Furthermore, with the increased dilutions of 1:50 and 1:10 anti-human Hsp72, CD36 expression significantly decreased ( $P<0.001$ , Figure 6.22A-B), to almost 2-fold ( $P<0.001$ ) and 3-fold ( $P<0.001$ ) with 42°C heat shocked monocytes (Figure 6.22A), compared to the 42°C sample without anti-human Hsp72 (Figure 6.22A). In addition, an almost 3-fold ( $P<0.001$ ) decrease and a 5-fold decrease ( $P<0.001$ ) was also observed with 46°C heat shocked monocytes compared to the 46°C control (Figure 6.23B). Treatment of cells with 1:10 dilution of anti-Hsp72 alone had no effect on CD36 expression compared to the 37°C control (Figure 6.22A-B). Heat shocked 46°C monocytes in combination with anti-human Hsp72 were found to be more effective than heat shocked 42°C monocytes in combination with anti-human Hsp72 at not increasing the surface expression of CD36 ( $P<0.001$ , Figure 6.22A-B).



**Figure 6.21: The effect of 42°C or 46°C heat shocked U937 monocytes on the induced expression of CD36 on U937 monocytes.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean temperatures from the untreated control through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



**Figure 6.22: The effect of 42°C (A) or 46°C (B) heat shocked U937 monocytes and/or anti-human Hsp72 (1:500-1:10 dilution) on the induced expression of CD36 on U937 monocytes.**

Data are presented as mean  $\pm$  SEM,  $n=3$ . Significance shown as difference between mean concentrations from the 37°C control or the 42°C or 46°C controls through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).

### **6.3.9 Migration of U937 macrophages and human leukocytes following incubation with bovine Hsp70, human Hsp72 or Hsp72 enriched supernatant.**

The experiments presented in figures 6.11 and 6.21 determined that human Hsp72 and Hsp72 enriched supernatant is able to stimulate an immune response from U937 macrophages. In order to explore this issue further, the following experiments were performed to determine whether bovine Hsp70, human Hsp72 or Hsp72 enriched supernatant can stimulate U937 macrophage and human leukocyte migration.

Migration of U937 macrophages and human leukocytes in response to HSPs are presented in Figures 6.23-6.26 and Tables 6.4-6.5.

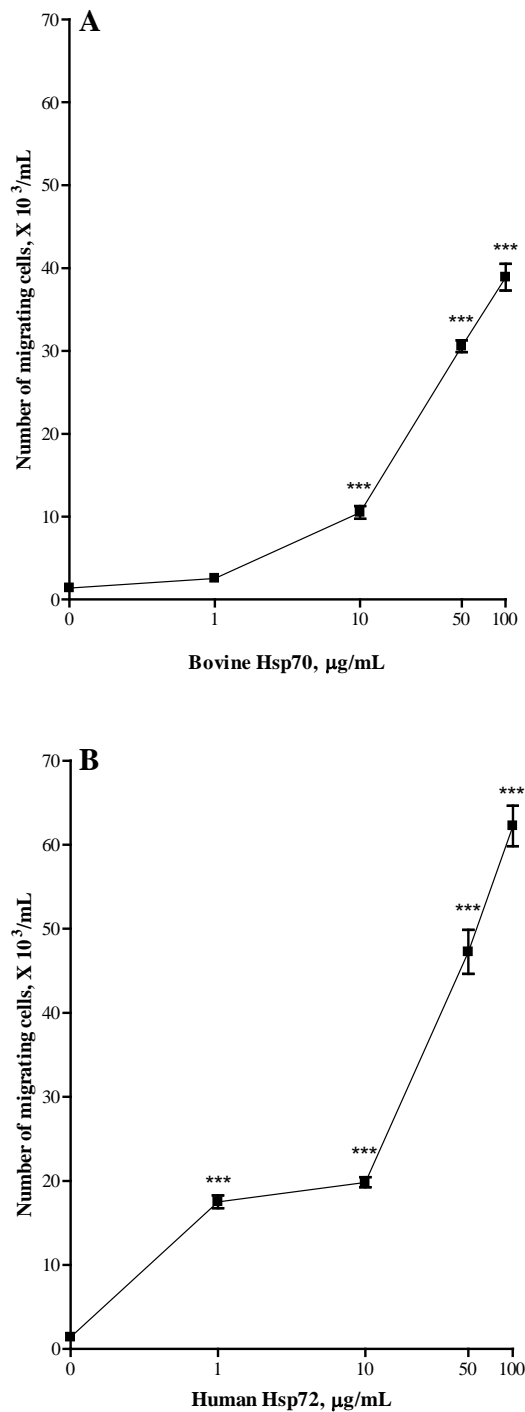
#### **6.3.9.1 Effect on cell migration of U937 macrophages following treatment with bovine Hsp70, human Hsp72 or Hsp72 enriched supernatant.**

Migration of U937 macrophages was significantly stimulated with 10 µg/mL bovine Hsp70 ( $P<0.001$ ) compared to the untreated control (Figure 6.23A). At concentrations below this (1 µg/mL) there was no significant difference from control (Figure 6.23A). In contrast, U937 macrophage migration was significantly stimulated with both 1 µg/mL ( $P<0.001$ ) and 10 µg/mL human Hsp72 ( $P<0.001$ ) compared to the untreated control (Figure 6.23B). The results suggest as the concentration of HSP increased the migration of U937 macrophages increased. A 16-fold increase ( $P<0.001$ ) was observed with 50 µg/mL bovine Hsp70 (Figure 6.23A) and an almost 25-fold increase ( $P<0.001$ ) with 50 µg/mL human Hsp72 (Figure 6.23B), again compared to the untreated control (Figure 6.23A-B). In addition, the migration of U937 macrophages increased almost 21-fold ( $P<0.001$ ) and 26-fold ( $P<0.001$ ) when incubated with the highest concentration of 100 µg/mL bovine Hsp70 (Figure 6.23A) and 100 µg/mL human Hsp72 (Figure 6.23B) compared to the untreated control (Figure 6.23A-B). Furthermore, migration of U937 macrophages towards LPS at a concentration of 100 ng/mL, also led to a significant 49-fold increase (1.4 to 69 cells/mL,  $P<0.001$ ) compared to the untreated control (Data not shown). Human Hsp72 was found to be more effective than bovine Hsp70 at

inducing the migration of U937 macrophages at concentrations of 50  $\mu\text{g/mL}$  ( $P<0.05$ ) and 100  $\mu\text{g/mL}$  ( $P<0.05$ , Figure 6.23A-B).

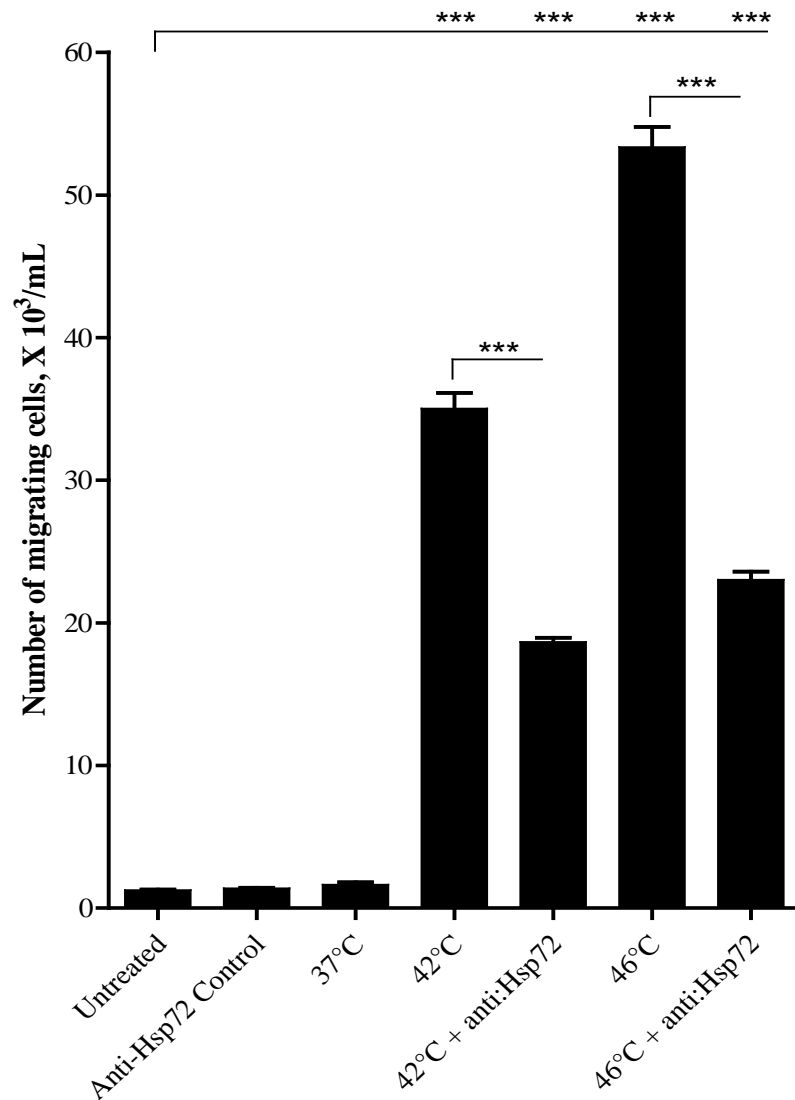
Migration of U937 macrophages was also significantly stimulated with Hsp72 enriched supernatant compared to the untreated control ( $P<0.001$ , Figure 6.24). A 20-fold increase ( $P<0.001$ ) was seen with Hsp72 released into 42°C supernatant and an almost 27-fold increase ( $P<0.001$ ) with Hsp72 released into 46°C supernatant compared to the untreated control (Figure 6.24). Furthermore, migration of U937 macrophages towards 100 ng/mL LPS also led to a significant 53-fold increase (1.2 to 67.8 cells/mL,  $P<0.001$ ) compared to the untreated control (Data not shown). There was no migration of U937 macrophages towards 37°C control supernatant. Hsp72 released into 46°C supernatant was found to be more effective than Hsp72 released into 42°C supernatant at stimulating the migration of U937 macrophages ( $P<0.001$ , Figure 6.24).

In contrast, the addition of 1:100 dilution anti-human Hsp72 to the 42°C or 46°C Hsp72 enriched supernatants for one hour at 37°C, prior to incubation with U937 monocytes for three hours, led to a significant decrease in the migration of U937 macrophages, compared to the 42°C ( $P<0.001$ , Figure 6.24) or 46°C samples without anti-human Hsp72 ( $P<0.001$ , Figure 6.24). Migration decreased almost 2-fold ( $P<0.001$ ) when stimulated with Hsp72 released into 42°C supernatant containing 1:100 anti-Hsp72 compared to the 42°C control (Figure 6.24), and again decreased almost 2-fold ( $P<0.001$ ) when stimulated with Hsp72 released into 46°C supernatant containing 1:100 anti-Hsp72 compared to the 46°C control (Figure 6.24).



**Figure 6.23: The effect on cell migration of U937 macrophages by bovine Hsp70 (A) or human Hsp72 (B).**

200  $\mu\text{L}$  of Hsp70 or Hsp72 (0-100  $\mu\text{g/mL}$ ) was applied to the lower chamber of a HTS transwell® permeable support system. U937 macrophages ( $5 \times 10^5$  cells/mL) were applied to the upper chamber and the plate was incubated for 4 hours. Cells within the lower chamber were counted. Data are presented as mean  $\pm$  SEM,  $n=3$ . \* indicates significant difference between mean concentrations compared to 0  $\mu\text{g/mL}$  control, through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



**Figure 6.24: The effect on cell migration of U937 macrophages by supernatant enriched with Hsp72 following a 42°C or 46°C heat shock treatment and/or anti-human Hsp72 (1:100 dilution).**

200  $\mu$ L of heat shocked supernatant was applied to the lower chamber of a HTS transwell® permeable support system with 1:100 dilution anti-human Hsp72. U937 macrophages ( $5 \times 10^5$  cells/mL) were applied to the upper chamber and the plate was incubated for 4 hours. Cells within the lower chamber were counted. Data are presented as mean  $\pm$  SEM,  $n=3$ . \* indicates significant difference between mean treatments compared to the untreated control or the 42°C or 46°C supernatants without anti-human Hsp72 through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\*\* ( $P<0.001$ ).



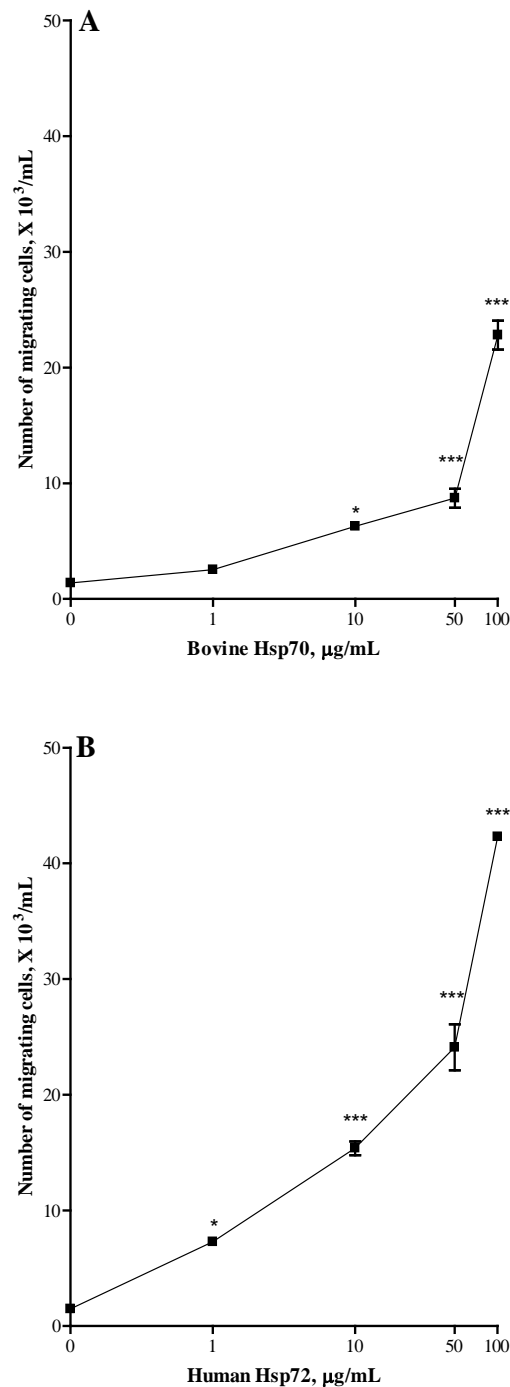
### **6.3.9.2 Effect on cell migration of human leukocytes following treatment with bovine Hsp70, human Hsp72 and Hsp72 enriched supernatant.**

Migration of human leukocytes was significantly stimulated with 10 µg/mL bovine Hsp70 ( $P<0.05$ ) compared to the untreated control (Figure 6.25A). At concentrations below this (1 µg/mL) there was no significant difference from control (Figure 6.25A). In contrast, human leukocyte migration was significantly increased with 1 µg/mL ( $P<0.05$ ) and 10 µg/mL ( $P<0.001$ ) human Hsp72 compared to the untreated control (Figure 6.25B). Similar to the results seen with U937 macrophages, as the concentration of HSP increased the migration of human leukocytes significantly increased ( $P<0.001$ , Figure 6.25A-B). Furthermore, migration of human leukocytes with FMLP at a concentration of 10 nM, also caused a significant 45-fold increase (1.4 to 62.2 cells/mL  $P<0.01$ ) compared to the untreated control (Data not shown). Human Hsp72 was found to be more effective than bovine Hsp70 at stimulating the migration of U937 macrophages at concentrations of 10 µg/mL ( $P<0.001$ ), 50 µg/mL ( $P<0.01$ ) and 100 µg/mL ( $P<0.001$ , Figure 6.25A-B). Due to the different leukocyte subtypes: neutrophils, lymphocytes and monocytes it was important to distinguish which leukocyte cells and what percentage were actually migrating as a result of HSP stimulation, thus the samples were analysed by flow cytometry (Table 6.4). The results revealed that neutrophils and lymphocytes respond most effectively to both bovine Hsp70 and human Hsp72 compared monocytes. Again, as the concentration of HSP increased the proportion of all three leukocyte subtypes migrating increased, compared to untreated controls (Table 6.4), which are consistent with previous findings (Figure 6.25). Neutrophil migration was increased at 100 µg/mL compared to the other cell types and the migration was the same as the FMLP control (Table 6.4).

Migration of human leukocytes was also significantly stimulated with Hsp72 enriched supernatant compared to the untreated control ( $P<0.001$ , Figure 6.26). A 12-fold ( $P<0.001$ ) and 22-fold ( $P<0.001$ ) increase was seen with Hsp72 released into 42°C supernatant and 46°C supernatant compared to the untreated control respectively (Figure 6.26). Furthermore, migration of human leukocytes towards 10 nM FMLP was significant with a 42-fold increase (1.5 to 63.2 cells/mL,  $P<0.001$ ) compared to the untreated control (Data not shown). There was no evidence of human leukocyte migration towards 37°C control supernatant. Hsp72

released into 46°C supernatant was found to be more effective than Hsp72 released into 42°C supernatant at stimulating the migration of human leukocytes ( $P<0.05$ , Figure 6.26).

As expected, the addition of 1:100 dilution anti-human Hsp72 to the 42°C or 46°C Hsp72 enriched supernatants for one hour at 37°C, prior to incubation with human leukocytes for three hours, led to a significant decrease in the migration of human leukocytes, compared to the 42°C ( $P<0.01$ , Figure 6.26) or 46°C samples without anti-human Hsp72 ( $P<0.01$ , Figure 6.26). Migration decreased almost 2-fold ( $P<0.01$ ) when stimulated with Hsp72 released into 42°C supernatant containing 1:100 anti-Hsp72 compared to the 42°C control (Figure 6.26), and again decreased almost 2-fold ( $P<0.01$ ) when stimulated with Hsp72 released into 46°C supernatant containing 1:100 anti-Hsp72 compared to the 46°C control (Figure 6.26). Flow cytometry data (Table 6.5) revealed that neutrophils and lymphocytes respond most effectively to Hsp72 enriched supernatant and to the positive control of FMLP compared to monocytes. Furthermore, when Hsp72 enriched supernatant was pre-incubated with anti-human Hsp72 (1:100 dilution), less leukocyte cells migrated (Table 6.5), which are consistent with previous findings (Figure 6.26). Neutrophil migration was increased with Hsp72 enriched supernatant compared to the other cell types and the migration was almost the same as the FMLP control (Table 6.5).



**Figure 6.25: The effect on cell migration of human leukocytes by bovine Hsp70 (A) or human Hsp72 (B).**

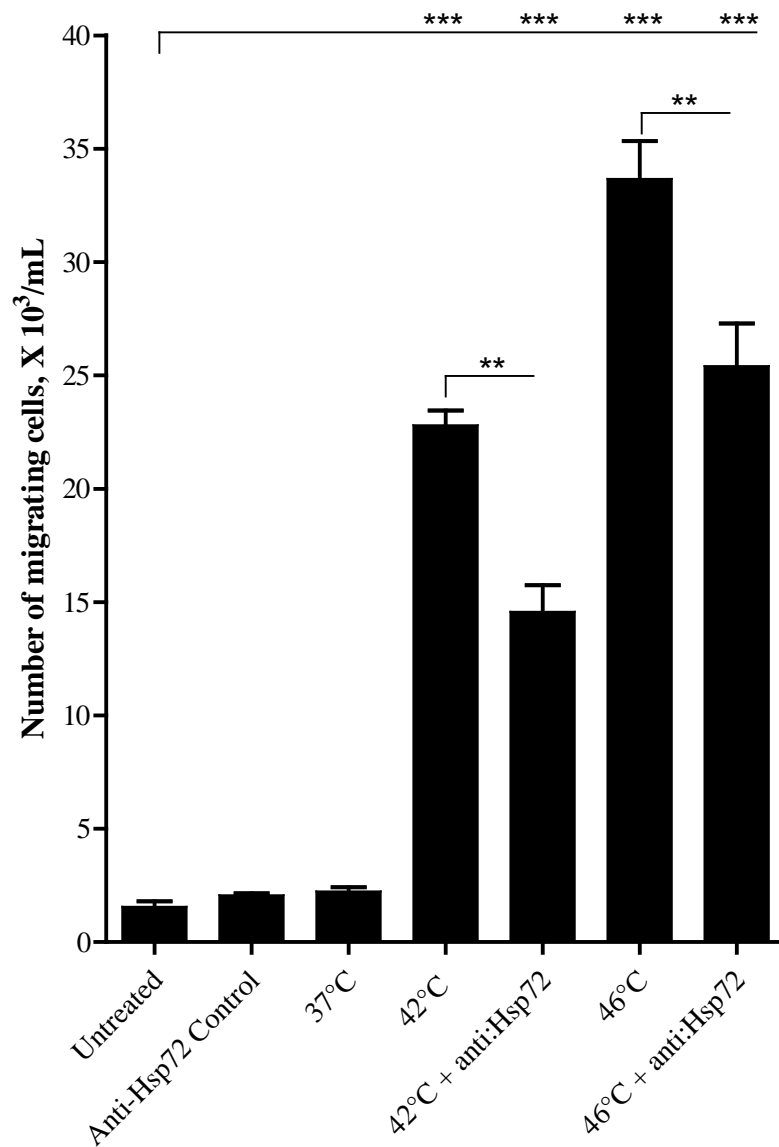
200  $\mu\text{L}$  of Hsp70 or Hsp72 (0-100  $\mu\text{g/mL}$ ) was applied to the lower chamber of a HTS transwell® permeable support system. Human leukocytes ( $3 \times 10^5$  cells/mL) were applied to the upper chamber and the plate was incubated for 2 hours. Cells within the lower chamber were counted. Data are presented as mean  $\pm$  SEM,  $n=3$ . \* indicates significant difference between mean concentrations compared to 0  $\mu\text{g/mL}$  control, through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \* ( $P<0.05$ ), \*\*\* ( $P<0.001$ ).

**Table 6.4: Migration of human leukocytes (neutrophils, lymphocytes and monocytes) using bovine Hsp70 or human Hsp72.**

200  $\mu$ L of bovine Hsp70 or human Hsp72 (0-100  $\mu$ g/mL) was applied to the lower chamber of a HTS transwell® permeable support system. Human leukocytes ( $3 \times 10^5$  cells/mL) were applied to the upper chamber and the plate was incubated for 2 hours. Migrated cells were counted and identified using antibodies to surface CD markers: CD3 (Lymphocytes) CD14 (Monocytes) and CD15 (Neutrophils) on the flow cytometer.

	<b>Treatment (<math>\mu</math>g/mL)</b>	<b>Percentage of lymphocytes</b>	<b>Percentage of monocytes</b>	<b>Percentage of neutrophils</b>
	<b>Untreated</b>	$0.1 \pm 0.0$	$0.0 \pm 0.0$	$3.2 \pm 1.1$
	<b>Positive Control</b>	$23.7 \pm 2.7$	$27.1 \pm 2.5$	$72 \pm 1.3$
<b>Bovine Hsp70</b>	<b>1</b>	$1.3 \pm 0.1$	$0.4 \pm 0.3$	$2.3 \pm 0.1$
	<b>10</b>	$4.5 \pm 1.3$	$0.9 \pm 0.2$	$13.8 \pm 1.6$
	<b>50</b>	$9.4 \pm 1.7$	$3.7 \pm 0.5$	$30.1 \pm 0.5$
	<b>100</b>	$11.9 \pm 1.1$	$10.9 \pm 0.8$	$55.3 \pm 1.4$
<b>Human Hsp72</b>	<b>1</b>	$2.5 \pm 0.7$	$1.8 \pm 1.2$	$10.4 \pm 0.9$
	<b>10</b>	$10.7 \pm 0.7$	$3.4 \pm 0.8$	$23.6 \pm 2.8$
	<b>50</b>	$14.7 \pm 1.1$	$8.9 \pm 1.6$	$43.2 \pm 4.8$
	<b>100</b>	$17.6 \pm 0.9$	$12.5 \pm 0.2$	$64.3 \pm 1.2$

Note: Data are presented as SEM, n=3. No treatment = HI-RPMI. Positive control = FMLP (10 nM). Seeding density  $3 \times 10^5$ . 10,000 events were recorded.



**Figure 6.26: The effect on cell migration of human leukocytes by supernatant enriched with Hsp72 following a 42°C or 46°C heat shock treatment and/or anti-human Hsp72 (1:100 dilution).**

200  $\mu$ L of heat shocked supernatant was applied to the lower chamber of a HTS transwell® permeable support system with 1:100 dilution anti-human Hsp72. Human leukocytes ( $3 \times 10^5$  cells/mL) were applied to the upper chamber and the plate was incubated for 2 hours. Cells within the lower chamber were counted. Data are presented as mean  $\pm$  SEM,  $n=3$ . \* indicates significant difference between mean treatments compared to untreated control or the 42°C or 46°C supernatants without anti-human Hsp72 through use of one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, \*\* ( $P<0.01$ ), \*\*\* ( $P<0.001$ ).

**Table 6.5: Migration of human leukocytes using supernatant enriched with Hsp72 following a 42°C or 46°C heat shock treatment, and/or anti-human Hsp72 (1:100 dilution).**

200  $\mu$ L of heat shocked supernatant was applied to the lower chamber of a HTS transwell® permeable support system with 1:100 dilution anti-human Hsp72. Human leukocytes ( $3 \times 10^5$  cells/mL) were applied to the upper chamber and the plate was incubated for 2 hours. Migrated cells were identified using antibodies to surface CD markers: CD3 (Lymphocytes), CD14 (Monocytes) and CD15 (Neutrophils).

<b>Treatment (°C)</b>	<b>Percentage of lymphocytes</b>	<b>Percentage of monocytes</b>	<b>Percentage of neutrophils</b>
<b>Untreated</b>	$1.9 \pm 0.1$	$0.5 \pm 0.2$	$1.9 \pm 0.5$
<b>Positive Control</b>	$23.7 \pm 2.7$	$27.1 \pm 2.5$	$72 \pm 1.3$
<b>37°C</b>	$0.7 \pm 0.2$	$0.8 \pm 0.1$	$1.9 \pm 0.1$
<b>42°C</b>	$6.6 \pm 0.9$	$2.7 \pm 0.2$	$39.4 \pm 0.4$
<b>42°C + anti-Hsp72</b>	$2.0 \pm 0.3$	$0.1 \pm 0.1$	$10.6 \pm 1.3$
<b>46°C</b>	$16.7 \pm 2.4$	$5.4 \pm 0.2$	$70.2 \pm 1.2$
<b>46°C + anti-Hsp72</b>	$6.2 \pm 1.5$	$0.7 \pm 0.1$	$32.9 \pm 3.3$

Note: Data are presented as SEM, n=3. No treatment = HI-RPMI. Positive control = FMLP (10 nM). Seeding density  $3 \times 10^5$ . 10,000 events were recorded

## **6.4 Discussion**

The aims of this chapter were to investigate: whether extracellularly administered (bovine Hsp70 or human Hsp72); intracellular (heat-induced); released extracellular HSP; or Hsp72 enriched (heat treated intracellular) supernatant were able to stimulate an immune response in U937 macrophages and human leukocytes.

### **6.4.1 Extracellular HSPs induce U937 monocyte differentiation.**

The first part of the study was performed in order to determine the effects of HSPs introduced into a U937 monocyte system. A number of different forms of HSPs: extracellular bovine Hsp70 and recombinant human Hsp72, Hsp72 enriched supernatant following a 42°C or 46°C heat shock treatment of U937 monocytes and heat shocked U937 monocytes with increased levels of intracellular Hsp72 were applied to U937 cell culture over a period of forty-eight hours. It was found that the addition of bovine or human HSPs in a concentration of 10, 50 or 100 µg/mL induced the expression of three surface monocyte/macrophage differentiation markers, CD36 (Figures 6.5-6.6), SR-A1 (Figure 6.7) and CD91 (Figure 6.8) in a time and dose-dependent manner. The concentrations employed in these studies appear high compared to reported extracellular HSP concentration, for example in the peripheral circulation of normal individuals 1131 ng/mL Hsp72 was observed in males whilst 2543 ng/mL Hsp72 was observed in females (Pockley et al. 1998). However, as shown by Basu et al. (2000b) lysate from one gram of tissue was estimated to yield microgram amounts, up to 200 µg/mL, of Hsp72 which upon cell lysis would result in a massive release of HSP into the extracellular environment (Basu et al. 2000b). The induction of the surface receptors was observed within the first hour of incubation at a concentration of 10 µg/mL (Figures 6.5-6.8), furthermore the up-regulation of the surface expression of CD36 was found to be higher than both SR-A1 and CD91 (Figures 6.5-6.6). The increased expression of CD36 could be due to the fact that it is a specific marker of macrophage activation and one of the first markers to be highly up-regulated during differentiation of pro-monocytes to monocytes and macrophages (Edelman et al. 1986; Armesilla et al. 1996, Huh et al. 1996; Panjwani et al. 2002). CD36 may act as a potential HSP-binding structure (Panjwani et al. 2002), and in this situation may be rapidly up-

regulated to recognise, bind and facilitate the internalisation of extracellular HSPs, which activate the cell they have bound to through interacting with CD36 and stimulating an immune response. Extracellularly administered HSPs were used as a model of Hsp72 release from necrotic cells as a result of tissue damage, and are suggested to function as a warning for the innate immune system which the body needs to recognise and respond to. In contrast, neither bovine Hsp70 nor recombinant human Hsp72 had any effect on the expression of CD14 (data not shown), which are consistent with the findings of Guzhova et al. (1998). A common criticism of *in vitro* studies of cellular responses to HSP is the use of recombinant HSP and LPS contamination. In order to determine whether LPS contamination was inducing the monocyte differentiation seen with extracellular HSPs, LPS was applied to U937 monocytes over a period of forty-eight hours. LPS was found to have no effect on monocyte differentiation compared to control cells (data not shown), suggesting the activity observed was not the result of LPS contamination. The increased expression of the surface markers CD36, SR-A1 and CD91 suggested extracellular HSPs induced the transformation of U937 monocytes into U937 macrophages, these results were confirmed through morphological studies (Figures 6.10, 6.15-6.16). The images revealed phenotypic characteristics of monocyte differentiation, such as increased cell size, clumping of cells, adherence to the well surface and the appearance of pseudopodia compared to control cells, which were not seen by Guzhova et al. (1998).

The experiments presented in the first part of this chapter revealed that the surface expression of CD36, SR-A1 and CD91 receptors are induced during monocyte to macrophage differentiation by extracellular HSPs, which are in agreement with previous studies (Huh et al. 1995; Huh et al. 1996, Binder et al. 2000; Basu et al. 2001). The up-regulation of these specific markers on both monocytes and macrophages may be a direct response of these cells to endogenous danger signals, which as mentioned, extracellular HSPs are proposed to act as when present in the extracellular environment (Asea et al. 2000b; Matzinger, 2002; Campisi & Fleshner, 2003a; Campisi et al. 2003b; Svensson et al. 2006), and thus are important recognition signals for the immune system, modulating an immune response and the inflammatory process. Macrophages are extremely efficient at recognising, binding and internalising foreign factors through a process called phagocytosis. CD36 cooperating with  $\alpha_v\beta_3$  receptors



and SR-A1 are both involved in phagocytosis, particularly of gram-negative and positive bacteria, fungi and inert particles (Wang et al. 2006), which suggests their induction during monocyte differentiation may increase macrophage clearance of apoptotic and necrotic cells (Savill et al. 1992). Indeed, Wang et al. (2006) demonstrated the presence of extracellular endotoxin free Hsp70 enhanced the rate and capacity of macrophage-mediated phagocytosis in the murine macrophage cell line RAW264.7 at six times the basal rate. Alternatively CD91, reported to be involved in receptor-mediated endocytosis, suggests the up-regulation of this receptor and also CD36 and SR-A1 may function as possible HSP-binding structures for extracellular HSPs, which has been previously reported (Binder et al. 2000; Berwin et al. 2003; Panjwani et al. 2002), and when bound facilitate the interactions of proteins or other cellular/chemical components with the extracellular membrane and aid extracellular HSP endocytosis.

The experiments performed to investigate the influence of U937 monocytes to the differentiation stimuli PMA at a concentration of 10 ng/mL also demonstrated a significant up-regulation of the surface markers CD36, SR-A1 and CD91 in a time-dependent manner. CD36 expression was up-regulated after a three hour culture (Figure 6.2), SR-A1 after a six hour culture (Figure 6.3) and CD91 after a twenty-four culture (Figure 6.4). The values for the three surface markers were substantially higher using PMA compared to both extracellular bovine and human HSPs at these time points. These results confirmed the transformation of U937 monocytes to U937 macrophages with PMA. However, Basta et al. (2001) suggests PMA impairs but does not completely abrogate monocyte to macrophage differentiation in primary blood monocytes rather than promote differentiation. This contrasts to the data presented here using cultured U937 monocytes as well as human monocytes, thus providing contradicting evidence to Basta et al. (2001).

The addition of the extracellular proteins prior to incubation with PMA significantly reduced the surface expression of these markers in a concentration-dependent manner when compared to cells stimulated with PMA alone (Figures 6.5-6.9). These results suggest the rapid up-regulation of the three surface markers by both treatments, extracellular HSPs and PMA occurred by similar pathways, such as the PKC pathway. This was confirmed by the experiments

shown in Figure 6.9 in which U937 monocytes were incubated with HSPs prior to incubation with increasing concentrations of PMA (0-20 ng/mL), which led to a significant decrease in the expression of CD36 compared to PMA or HSP alone. These results are in agreement with another other study (Guzhova et al. 1998). The most probable pathway is PKC which is utilized by PMA during differentiation of monocytes to macrophages (Zhang et al. 1995). The results presented in Figure 6.9 suggest that the administered HSPs may bind to and occupy surface structures used normally by PMA such as PS, making them inaccessible to PMA and potentially inhibiting PKC pathways. However, PS should only be present on the surface of senescent cells and the addition of extracellular HSPs to U937 monocytes did not affect their viability (as demonstrated in chapter 4), suggesting HSPs and PMA may interact with an alternative structure to PS on the cell surface. The potential interaction of HSPs with the cell surface has been previously discussed in chapters' three to five and several studies have demonstrated this (Alder et al. 1990; Negulyaev et al. 1996; Arispe et al. 2004; Vega et al. 2008; Schilling et al. 2009). These data establish that extracellularly administered HSPs negatively regulate PMA-dependent monocyte to macrophage differentiation.

#### **6.4.2 Heat shock derived Hsp72 induces U937 monocyte differentiation.**

To test whether HSPs up-regulated in response to a heat shock treatment and released into the supernatant could also influence macrophage differentiation, a heat treatment protocol was designed based on the observations seen in chapter three. A one hour heat shock at 42°C or 46°C induced the up-regulation (as demonstrated in chapter 3), and release of Hsp72 which was confirmed by an Hsp72 ELISA (Table 6.2). Three different heat treatments were then applied to U937 monocytes: U937 monocytes were heat shocked directly then incubated with PMA, or U937 monocytes were incubated with Hsp72 enriched supernatant released from 42°C or 46°C U937 monocytes or viable U937 monocytes were incubated directly with apoptotic and necrotic U937 monocytes. A direct heat shock had no effect on the expression of CD36 (Figure 6.12) or SR-A1 (Figure 6.13) surface markers, but led to an increase in the expression of CD91 (Figure 6.14). A possible reason for the lack of an effect of heat shock on the expression of CD36 and SR-A1, could be due to the fact that the heat shock treatment had

up-regulated intracellular Hsp72 (as demonstrated in chapter 3), which functions to protect cells from heat shock induced apoptosis (Garrido et al. 2001; Stankiewicz et al. 2005). As a result, the internal derived signal(s) would have suggested the cells to have adapted to changes in their environment and induced a state of acquired thermotolerance. Therefore, there would have been no functional relevance up-regulating CD36 or SR-A1 receptors because they facilitate the clearance of damaged cellular components *via* phagocytosis inducing an immune response. In this situation, this process was not required since intracellular Hsp72 would have prevented protein denaturation, or irreversible aggregation and damage to cellular structures. On the other hand, the up-regulation of CD91 may have been induced to facilitate in receptor-mediated endocytosis of bound cellular/chemical components induced during the heat treatment protocol, however this would require further investigation, as would the expression of other cell surface receptors. However, flow cytometry data revealed that when these cells were incubated with PMA for three hours following the heat treatment protocol there was an up-regulation of the surface expressions of CD36, SR-A1 and CD91 compared to heat shock treatment alone. This was possibly due to the fact that these cells had now been exposed to two stressors, up-regulating intracellular Hsp72 (as determined in chapter 3) and stimulating membrane localisation of Hsp72 (as determined in chapter 3), or release of host-derived Hsp72 into the extracellular environment. Leakage of intracellular Hsp72, which as suggested acts as a danger signal, may have stimulated the immune system and thus initiated the uptake of dead cells, stress damaged cells or their debris *via* CD36 or SR-A1 receptors, stimulating phagocytosis. Alternatively, the increase in expression of the surface receptors could be due to the fact that intracellular induced Hsp72 did not interfere with the PKC pathway and the effects of PMA, in particular with the membrane lipid PS as seen with extracellularly administered HSPs. Due to the heat treatment protocol, PS may have translocated from the inner leaflet of the plasma membrane to the outer leaflet in a mechanism that commonly occurs in the early stages of apoptosis, thus enhancing the effects of PMA and stimulating the expression of the surface markers CD36, SR-A1 and CD91, again in order to remove the cell debris or facilitate the binding and endocytosis of HSPs, enabling further signalling.

To fully elucidate the effects of HSPs derived from heat shock it was investigated whether these effects could also occur *ex vivo*. Thus human monocytes were isolated from healthy volunteers and incubated for one hour at 42°C or 46°C in combination with PMA, which was found to significantly induce the up-regulation of the surface expression of CD36 (Figure 6.17), concluding that the effects of HSPs are not limited to cultured cells, which again disagrees with the findings of Basta et al. (2001), but agrees with the findings of Panjwani et al. (2002). Apoptotic and necrotic cells directly applied to U937 monocytes (Figure 6.21), in addition to Hsp72 enriched supernatant were also found to significantly induce the surface expression of CD36 (Figure 6.18). Monocytes and macrophages function in the removal of physiologically aberrant cells, they are also known to bind Hsp72 (Delneste et al. 2002), thus CD36 may be up-regulated in this situation as an immune response to the endogenous self-components, in order to recognise, then bind the extracellular HSPs, or the apoptotic cells and necrotic debris, internalise them through endocytosis or process their removal *via* phagocytosis (Tani et al. 2009). This expression was also increased following incubation with Brij®-98, a nonionic surfactant which is reported to isolate fractions of the lipid membrane, suggesting Hsp72 may be contained within detergent-soluble membrane vesicles known as exosomes or lipid rafts when released from heat shocked cells. These results are in agreement with previous findings (Bausero et al. 2005; Clayton et al. 2005; Gastpar et al. 2005; Lancaster & Febbraio, 2005). Further confirmation of this role was demonstrated by the increase in expression of CD36 by Hsp72 enriched supernatant which was blocked with anti-human Hsp72, concluding that Hsp72 was indeed causing the increased surface expression of CD36.

#### **6.4.3 Cytokine response.**

It was also demonstrated that human Hsp72 (10 µg/mL) incubated with U937 monocytes over a period of forty-eight hours was capable of initiating an immune response through the increased secretion of pro- and anti-inflammatory cytokines, TNF- $\alpha$  (Figure 6.11) and IL10 (Figure 6.11). This is in agreement with other studies (Wang et al. 2001; Kakimura et al. 2002; Wang et al. 2005b; Svensson et al. 2006; Wang et al. 2006). Whilst Hsp72 enriched supernatant released from 42°C or 46°C heat shocked U937 monocytes, caused a reduced

cytokine secretion when compared to human Hsp72. In fact it increased the secretion of TNF- $\alpha$  (Table 6.3) only. Modulating an immune response suggests a function for HSPs in inflammatory control, for example aiding the tissue repair process after inflammation. Kovalchin et al. (2006) demonstrated extracellular HSPs to actively promote tissue repair in murine models of wound healing by promoting phagocytosis. This suggests a possible role for HSPs in the treatment of wound healing in diseases and traumas including diabetes, sepsis and burns. Furthermore, it was also shown that human Hsp72 alone and Hsp72 enriched supernatant could stimulate the migration of both macrophages and human leukocytes, suggesting a role for HSPs as a chemoattractant, stimulating an immune response, leading to phagocytosis of apoptotic cells or necrotic debris. This effect was blocked with anti-human Hsp72 in a dose-dependent manner however the effect was not completely abrogated.

The results observed in this chapter conclude that HSPs whether bovine Hsp70, human Hsp72, intracellular (heat-induced) or released extracellular Hsp72 from heat treated monocytes are able to induce monocyte differentiation, modulate an immune response through stimulating pro- and anti-inflammatory cytokine release and is a chemoattractant. Therefore Hsp72 is clearly capable of interacting at several different levels with cells of the innate and adaptive immune systems and may function in the regulation of inflammatory responses.

## Chapter 7

### General Discussion

HSPs are intracellular molecular chaperones whose primary function is to control the folding, transport and degradation of a variety of naïve and denatured polypeptides and proteins, under both normal physiological conditions and following exposure to potentially harmful stress stimuli (Gething & Sambrook, 1992). In recent years however, it has also been demonstrated that many HSPs are present in the extracellular environment, both basally and in response to cellular stress, either in the extracellular milieu (Tytell et al. 1986; Hightower & Guidon, 1989; Pockley et al. 1998; Wright et al. 2000), on the cell surface or within the cellular membrane of tumour (Multhoff et al. 1995), and virally-infected (Chouchane et al. 1994) cells. Importantly, several studies have established that this release is the result of an active secretory process despite lacking a peptide leader sequence targeting secretion (Broquet et al. 2003; Hunter-Lavin et al. 2004b; Clayton et al. 2005; Davis et al. 2006; Mambula & Calderwood, 2006a), as well as traditional non-specific processes such as injury, tissue damage or cell lysis (Basu et al. 2000b). The functions of extracellular HSPs are not completely elucidated, but reports have shown biological activity in cyto-protection, inter-cellular signalling, and also interactions with the innate and adaptive immune responses (Multhoff, Botzler & Issels, 1998; Srivastava, 2002a; Millar et al. 2003). The present study ultimately aimed to investigate the possible cyto-protective and immune regulatory roles of extracellularly administered HSPs applied directly to the cultured cell lines, human monocytic U937 cells and U937 macrophages, and human peripheral blood cells, leukocytes and erythrocytes in response to various stress stimuli, and the mechanism(s) by which extracellular HSPs may exert these potential effects. The general discussion will consider the following in respect to extracellular HSPs, in particular Hsp72:

- Cyto-protection
- Membrane localisation
- Receptor interactions
- Immune regulation

## 7.1 Cyto-protection

Cellular processes can be compromised during exposure of cells to environmental stress such as a significant increase in temperature. The expression of internal HSP is a normal response to minimise cell injury and aid survival. Although many aspects of the stress response have already been elucidated in relation to extreme temperature as a stressor in almost all organisms, little is known about the location and physiological function of HSPs in specific cells under these conditions. Thus, the first objective of this thesis was to investigate the effects of an increase in temperature on apoptotic and necrotic cell death and the induced whole cell expression and localisation of a number of HSPs: Hsp27, Hsp60, Hsp72 and Hsp90 *in vitro* and *ex vivo* in cultured and primary cell lines compared to control cells. As expected (chapter three), it was found that an elevation of temperature had a profound effect on the proportion of both apoptotic cells at temperatures above 40.8°C and necrotic cells at temperatures above 44.4°C, and also on the levels of whole cell Hsp72 located in the cytosol of permeabilized U937 cells and human leukocytes exposed to the sub-lethal temperature of 42°C for one hour compared to controls. Similarly, human leukocytes exposed to this sub-lethal temperature also up-regulated Hsp27, Hsp60 and Hsp90 located in the cytosol compared to controls but the levels were significantly less than Hsp72 and therefore it may be proposed that a longer exposure or increased temperature would achieve similar levels to Hsp72 as previously described (Martin, Horwich & Hartl, 1992; Arrigo et al. 1998; Fortugno et al. 2003). The up-regulation of these proteins would support protein re-folding and intracellular protein trafficking, both of which are severely affected as a consequence of an increase in temperature, and also inhibit the process of heat-induced apoptosis as previously described (Garrido et al. 2001; Stankiewicz et al. 2005; Schmitt et al. 2007). These results provided further evidence for the induction of HSPs during the stress response. In contrast, a significant decrease of whole cell HSP levels: Hsp27, Hsp72 and Hsp90 was observed in the cytosol of permeabilized U937 cells and human leukocytes following a three hour recovery at 37°C compared to heat shock controls, with the exception of Hsp60. The decrease of whole cell HSPs may be explained by a potential re-distribution of the cytosolic proteins to the cell membrane (section 7.2), or due to the possibility that a partial denaturation of the cellular content

and/or cell death occurred following the exposure of cells to an increase of temperature at 42°C. On the other hand, levels of whole cell Hsp60 which increased in the cytosol after recovery may have been internally restored at three hour post treatment, potentially correlating with the up-regulation of *HSP* genes induced by the increase in temperature. This suggests that Hsp60 has an essential role inside cells, possibly facilitating the degradation of damaged proteins *via* the proteasome (Becker & Craig, 1994; Hartl, 1996; Bukau & Horwich, 1998) induced by heat-stress, again providing further evidence for the induction of HSPs during the stress response.

Although HSPs are classically regarded as being intracellular proteins with undisputed roles in cell survival and protection, there is a growing amount of literature providing evidence that specific HSPs can be released from stressed cells into the extracellular environment (Guzhova et al. 2001; Barreto et al. 2003; Hunter-Lavin et al. 2004b; Davis et al. 2006). A potential biological function for this accumulation of HSP outside the cell may be cyto-protection (Guzhova et al. 1998; Guzhova et al. 2001). This property of extracellular HSPs is suggested to be related to their ability to bind to, and be internalised by, cells, possibly in a receptor-dependent manner (section 7.3). The second objective of this thesis therefore was to determine whether extracellular bovine Hsp70 or human Hsp72 could bind to the cell surface, cross the plasma membrane and be taken up by U937 and human peripheral blood cells. Extracellularly administered HSPs bound to the cell surface and were internalised in a cell-type specific, time- and concentration-dependent manner (chapter four). The results found the binding and internalisation of extracellular HSPs increased with increasing concentrations, up to 50 µg/mL, and were even detected when cells were incubated with levels as low as 5 µg/mL, which occurred over a time course extending up to four hours. These concentrations are found in the circulation (0.1-5 µg/mL), which provides evidence for a potential functional relevance *in vivo* (Pockley et al. 1998; Basu et al. 2000b; Rea et al. 2001; Njemini et al. 2003). When added exogenously to cells, these proteins were found to bind relatively quickly in all cell types in under an hour, specifically within twenty (U937 cells) and forty minutes (human leukocytes), reaching saturation within two hours. The binding of extracellular HSPs to the cell surface may affect



surface structures and alter their permeability characteristics, and by this potentially stimulate various signalling systems, subsequently initiating internalisation (Vega et al. 2010). Within the studied time frame, internalisation of the proteins occurred as expected, however the internalisation was much slower, taking over an hour and maximal internalisation was not achieved until three to five hours after the addition of extracellular HSPs. Further studies would investigate a longer incubation period perhaps up to twenty-four hours to fully elucidate the internalisation of extracellular HSPs. It may be proposed following internalisation, the proteins diffused throughout the cytoplasm but not in the nucleus, since there was no evidence of a co-localisation of the HSPs studied with the nucleus. However, it may be speculated that a longer incubation could result in the localisation of extracellular HSPs in the nucleus as previously described (Arrigo et al. 1998; Fujihara & Nadler, 1999). The binding and internalisation of extracellularly administered HSPs may function to offer cellular protection through stabilising the cell membrane during thermal insults, or they may even function like intracellularly expressed HSPs inhibiting the process of apoptosis. Despite the increased levels of HSPs inside cells, it cannot be completely elucidated that the extracellularly administered HSPs were actually internalised. It can also be speculated that the binding of HSPs to the cell membrane initiated cell signalling which induced internal HSP gene expression and subsequently intracellular HSPs, this would need to be confirmed by gene expression analysis. If this were the case, it does appear to be highly unlikely because the converse has actually shown to be the case. Intracellular Hsp70 gene expression is decreased by excess intracellular Hsp70, as it sequesters any free HSF1 (Gething & Sambrook, 1992; Morimoto, Sarge & Abravaya, 1992). These hypotheses require further investigation, whereby the supernatant would need to be analysed by an ELISA measuring the levels of HSPs compared to control to confirm the uptake of these proteins, or through the use of fluorescently labelled extracellularly administered HSPs and intracellular analysis using flow cytometry. Evidence of the uptake of Hsp70 has been previously suggested. Guzhova and colleagues, (1998) revealed biotinylated Hsp70/Hsc70 internalisation in U937 cells within one hour of incubation which is in agreement with the results presented in this study. Whilst Fujihara & Nadler, (1999) showed uptake of Hsp70 into the cytoplasm and nucleus of monocytes but not

lymphocytes, and also limited uptake by U937 cells but with higher concentrations than the ones employed in this study, located in the cytosol but not in the nucleus, which again is in agreement with this study. The results of the present study have shown extracellular HSPs whether bovine or human, bound to, and were internalised in a cultured cell line, U937, but more importantly this effect was also shown in primary peripheral human leukocytes. Furthermore, unlike for example spinal sensory neurones (Houenou et al. 1996; Robinson et al. 2005), neuroblastoma cells (Guzhova et al. 2001) and motor neurones (Tidwell et al. 2004) which are all deficient in Hsp70, both U937 cells and human leukocytes express Hsp70 basally and are capable of up-regulating Hsp72 in response to cellular stress. Despite this, these cells were able to bind, and internalise extracellular HSPs even in non-stressed conditions suggesting important roles for extracellular HSPs not only in response to conditions of stress but under normal physiological conditions.

The fact that human leukocytes could bind extracellular HSPs (chapter four), led to the investigation as to whether extracellular HSPs could also bind to the cell surface of enucleate human erythrocytes and internalise, despite the fact that these cells cannot produce their own HSPs. It was established (chapter five) that non-permeabilized erythrocytes could indeed bind extracellular HSPs in a time- and concentration-dependent manner. In fact, analysis of the kinetics of HSP binding indicated it to be rapid, revealing a typical rectangular hyperbola giving a  $KD$  of  $7.08 \pm 1.43 \mu\text{g/mL}$  for human Hsp72 and suggested a receptor-mediated interaction (section 7.3). However, the Western blot of intracellular protein suggests that Hsp72 was not internalised in one hour. Alternatively it may be bound to the cytosolic side of the plasma membrane. This is in agreement with Johnson & Tytell, (1993) who indicated that exogenous Hsc70 becomes associated with the cell surface, but is not internalised in primary culture of rabbit arterial smooth muscle cells. It could be speculated that a longer incubation may result in an internalisation of extracellular Hsp72 into the cytosol.

The function of internalising extracellular HSPs *in vivo* proposes the process to have relevance in cell-to-cell communication, perhaps as a means of transferring cellular protection from environmental stresses by regulating transcription. HSPs

released from cells into the milieu *via* necrotic cell death, may interact with and provide cells with cellular protection through binding and internalisation. There is a growing amount of evidence which suggests extracellular HSPs *in vivo* and externally supplied HSPs *in vitro* can interact with cells to promote stress tolerance (Johnson et al. 1990; Houenou et al. 1996; Guzhova et al. 1998; Guzhova et al. 2001; Yu et al. 2001; Tidwell et al. 2004). It appears important therefore, that upon binding and being internalised by a cell, extracellular HSPs retain the capacity to protect cells from a variety of stressful conditions. Previously, it has been shown that, cells which have received a mild non-lethal temperature treatment exhibited significantly higher survival rates after a second temperature challenge that would otherwise be lethal because of increased intracellular HSPs, a phenomenon referred to as thermotolerance (Parsell & Lindquist, 1993). It was therefore hypothesised (third objective of this thesis) that HSPs in the extracellular milieu, released from stressed damaged cells, (for the purpose of this thesis externally administered to cells), could influence and rescue cells from apoptotic and necrotic cell death in a similar action to the process of thermotolerance, hence providing cells with cyto-protection. In contrast to previous studies, this thesis used two different preparations of the 70-KD HSPs: bovine Hsp70 and human Hsp72 and found that they had similar effects on U937 cells, human leukocytes and erythrocyte survival after heat shock treatment. It is noteworthy that a control protein, BSA had no effect. Chapter four reports the finding that pre-conditioning with extracellular bovine Hsp70 induced tolerance to an elevation of temperature in both U937 cells *in vitro* and human leukocytes *ex vivo*. Specifically this protein changed the pattern of apoptosis and necrosis in these cells, preventing cell death following heat shock treatment. These results suggest cells become resistant to stress when their intracellular HSPs are supplemented by the administration of extracellular HSPs, consistent with the theory of thermotolerance. It also proposes that extracellular HSPs may function in a similar manner to intracellular HSPs inhibiting pro-apoptotic signalling pathways or promote maintenance of survival pathways, stabilise damaged membranes or preserve the integrity of the cell membrane (Vigh et al. 1998), or even, once internalised, enter intracellular compartments in which intracellular produced HSPs reside and function. *In vivo* extracellular HSPs may do this by facilitating the internalisation of molecules, such as critical

nutrients including transferrin, essential for the uptake of iron (Vega et al. 2010), which function in cell repair and facilitates the return to cellular homeostasis. This suggests a unique functional role in the context of cyto-protection for low concentrations of extracellular HSPs and may have therapeutic potential in the prevention of cell death as a result of trauma or disease.

Although it is generally assumed that a cell must produce its own HSPs to be protected by them, there is evidence that HSPs can pass from cell-to-cell and improve the resistance of cells to stress which appear to be deficient in the HSP stress response (Tytell 1986; Guzhova et al. 2001). This theory was investigated *ex vivo* using enucleate human erythrocytes which are unable to produce HSPs. The results of chapter five revealed a pre-treatment of erythrocytes with extracellular HSPs prevented heat shock induced, as well as osmotic and uric acid induced haemolysis, suggesting HSPs have the ability to stabilise the erythrocyte membrane physical structure. This proposes *in vivo* HSPs released from other circulatory cells or cells which line the interior surface of blood vessels into the extracellular milieu, potentially human leukocytes or endothelial cells, may act as a stimulus for the immune response and function to preserve erythrocyte function under stressful conditions to ensure adequate delivery of oxygen to the tissues. Thus, HSP release from human leukocytes may be a natural property which erythrocytes depend upon for their survival and may also have positive effects on other cells with low HSPs. The results presented in chapters four and five have hinted at the hypothesis that some of the protection from environmental, and physiological stress induced by extracellular HSPs is derived from membrane stabilisation. This and the fact that protection from necrosis is also observed suggests that the protective activities of HSPs, in particular Hsp72 are multi-faceted.

## **7.2 Membrane localisation**

The effect of an increase in temperature is not limited to the up-regulation of intracellular HSPs established here (chapter three) and in earlier reports (Parsell & Lindquist, 1993; Morimoto & Santoro, 1998). This study also provides evidence of the translocation of HSPs from the cytosol to the cell surface. Following an hour treatment of non-permeabilized cells with the sub-lethal temperature of 42°C, intracellular HSPs including Hsp27, Hsp60, Hsp72 and

Hsp90 became exposed at the cell surface and which in the case of Hsp72 and Hsp90 increased following a period of recovery at 37°C. This suggests that both these proteins, which are co-chaperones along with Hsp40, are essential in the functioning and/or protection of the cell during conditions of elevated temperature stress potentially through cell-to-cell signalling with neighbouring cells, or through facilitating the stabilisation of the cell membrane. However, it may actually reflect the opposite of this, the presence of these HSPs on the cell membrane could possibly be part of a mechanism to limit the presence of HSPs inside cells, because despite being protective in the short term there is evidence that in the long term, the presence of HSPs within cells could be cytotoxic (Feder et al. 1992; Arispe et al. 2004). Thus, the potential release of HSPs from cells could be an additional mechanism to avoid any cytotoxic effects of the excessive intracellular HSPs. However, with regard to the short time scales employed in this study this hypothesis would appear to be highly unlikely. It may also be proposed that the cell surface expression of HSPs during conditions of stress and recovery act as 'lifebelts' for adjacent cells which suffer from a deficiency of intracellular HSPs or are unable to mount a successful stress response. These cells may therefore bind and take up surface expressed and/or released HSPs, internalising them and thus providing these cells with cyto-protection. Both these situations may be the case for Hsp27 and Hsp60 because in contrast to the other proteins studied, their levels decreased after a period of recovery, possibly suggesting their release from the cell membrane into the culture media, which would require further investigation using an ELISA. The fact that the levels of these surface expressed proteins decreased during recovery may also reflect the possibility that these proteins were unable to aid cell survival during the stress response. Indeed, Hsp27 has an established role of regulating structural integrity and membrane stability (Arrigo et al. 2002; Kostenko et al. 2009); however the heat shock treatment may have been too much for these proteins and were potentially released. It may therefore be proposed that *in vivo* the interaction of HSPs with the plasma membrane acts as a platform for their release into the extracellular environment, which is an area for future study. It has been previously suggested that the interaction of Hsp72 with cell membrane lipids is unique phenomena because other HSPs including Hsp27, Hsp60 and Hsp90 do not associate with artificial membranes (Vega et al. 2008), yet this study clearly

demonstrated an association of the HSPs studied with the plasma membrane *via* a receptor interaction, membrane-bound following heat stress at 42°C and a period of recovery at 37°C.

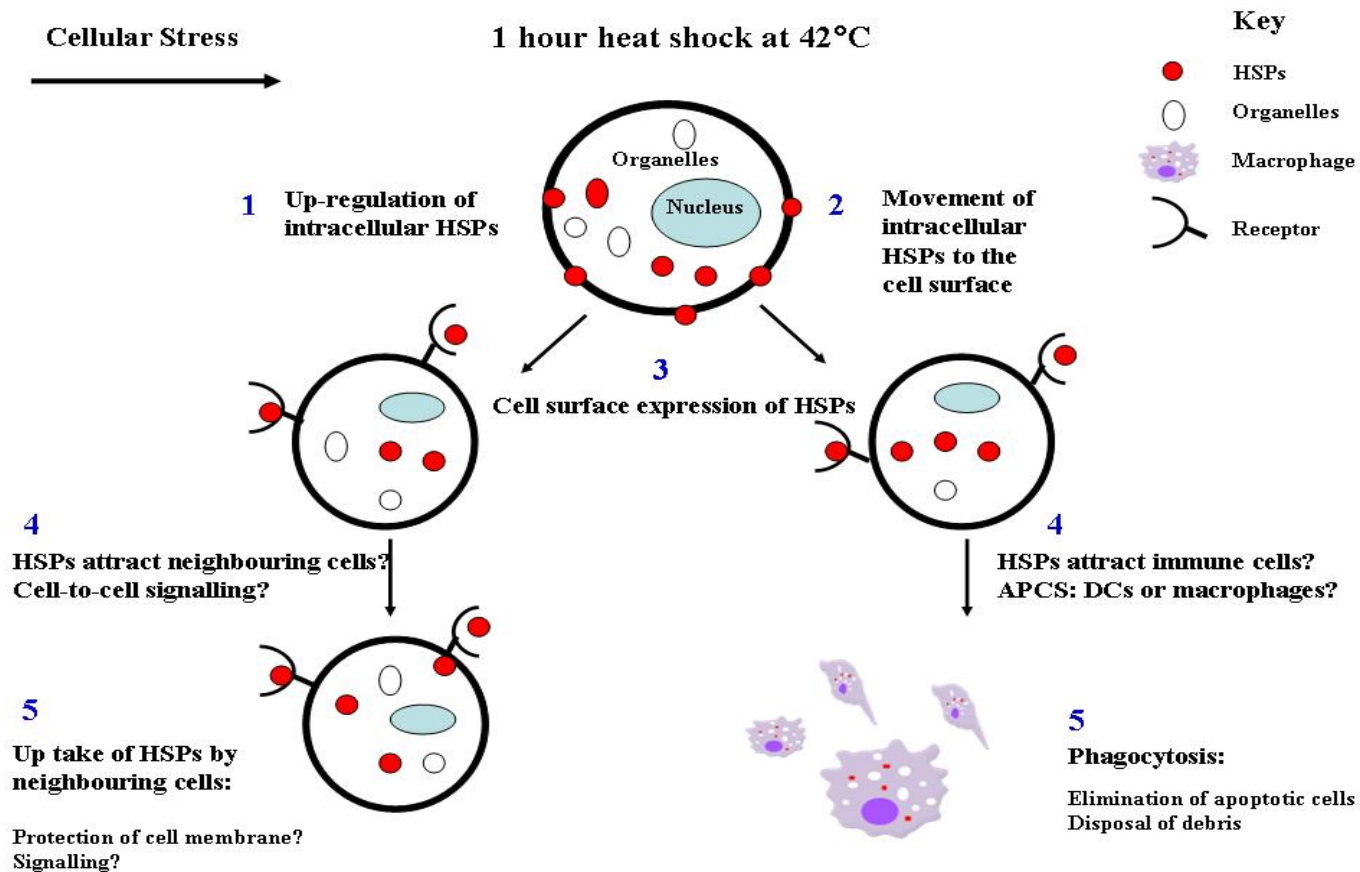
Multhoff et al. (2001), confirmed by Korbelik et al. (2005) and Vega et al. (2008) have shown that the C-terminal domain of Hsp72, specifically a 14-mer sequence termed TKD, is exposed to the extracellular milieu through extruding the plasma membrane when this molecule is expressed at the cell surface, whilst the remaining two domains are unexposed within the membrane, presumably located on the cytosolic side. In accordance with this, Hsp72 only, also became incorporated into the lipid bilayer under stress and increased following a period of recovery, detected with an antibody (cm.Hsp70.1) specific for the TKD epitope, detecting membrane-embedded Hsp72 on neutrophils and lymphocytes and to a lesser degree on monocytes. It has been proposed that Hsp72 inserts into the plasma membrane through its ability to assemble into low-order oligomers when it is bound to and chaperoning unfolded polypeptides after stress (Arispe et al. 2004). Indeed, the C-terminal domain of Hsp72 functions in peptide binding and regulation, whereby Hsp72 proteins interact with chaperoned client peptides and endogenous peptides which are a potential source of cellular damage for the surveillance of phagocytosing cells (Tavaria et al. 1996). It has been previously suggested that cells expressing surface HSPs are preferentially attacked by immune cells, such as macrophages and DCs and are eliminated (Xu et al. 1994; Török et al. 1997). Hence, it may be proposed *in vivo* that membrane located Hsp72, chaperoning damaged cellular proteins through its substrate-binding domain as a result of an increase of temperature, become exposed to immune surveillance cells and are recognised, engulfed and ingested *via* phagocytosis. This activity would be similar to that seen in tumour cells expressing surface HSPs which are also preferentially attacked by immune cells such as CD94<sup>+</sup> NK cells and are eliminated *in vivo* (Barreto et al. 2003; Gross et al. 2003a; Gastpar et al. 2005). The surface expression of HSPs and their effects are summarised in figure 7.1.

It has been generally recognised that the membrane localisation of HSPs (membrane-bound or embedded) are restricted to transformed (Multhoff et al. 1995) and virally infected cells (Chouchane et al. 1994), and that in normal cells Hsp72 is strictly localised within the cell except for spermatogenic cells (Dix et

al. 1996; Hartmann, Lingwood & Reidl, 2001; Gehrmann et al. 2008). Yet, this study provides evidence for the first time of the surface expression of HSPs under basal conditions on normal human peripheral blood cells including leukocytes (chapter three), but not on erythrocytes (chapter five) and also in response to conditions of stress (chapter three). This suggests that the phenomenon of surface expressed HSPs is not limited to tumour or virally infected cells. The function of HSPs localised in the outer cellular membrane is not well understood, but it has been suggested to have functional roles in processes such as cell maturation and migration (a role confirmed in chapter six) as well as being associated with the capacity of these proteins to stabilise the lipid membrane (Vigh et al. 2007), through associating with long length fatty acid chains (Guidon & Hightower, 1986), or certain surface receptors (Kurucz, Tombor & Prechl, 1999), which may indeed be the case under basal conditions. However, this study (chapter three) identified that the inducible expression of HSPs on the surface of these cells corresponded with a particularly pronounced expression on those cells undergoing heat-induced apoptosis or necrosis, therefore not preserving their integrity or re-establishing the fluidity and bilayer stability of the membrane during thermal insults. This study may therefore suggest that an increase of cells expressing cell surface HSPs in association with lipids such as PS reflects membrane damage and/or cell death during the stress response. Elevated surface expressions of HSPs in cells undergoing apoptosis have been reported previously (Poccia et al. 1996; Sapozhnikov et al. 1999; Sapozhnikov et al. 2002; Korbelik et al. 2005). Future studies would be required to examine whether the translocation of intracellular HSPs to the cell membrane is an exclusive effect of an increase in temperature. Previously, Vega and co-workers (2008) have found treatment of HepG2 cells with geldanamycin as well as heat shock, induced the cell surface expression of Hsp70, suggesting that the translocation of HSPs to the cell membrane as demonstrated in chapter three is not exclusive to an increase of temperature but may be a natural property of apoptotic cells. It has been generally regarded that the up-regulation of intracellular HSPs, especially Hsp72 results in the inhibition of programmed cell death through blocking both the intrinsic and extrinsic pathways of caspase-dependent apoptosis (chapter three). However, this inhibitory effect may be hindered by heat-induced translocation of HSPs to the cell surface. Thus, the

results of this study have determined, depending on their location and cell type: normal or stressed cells, HSPs can either protect cells from or promote apoptotic or necrotic cell death and subsequently phagocytosis.





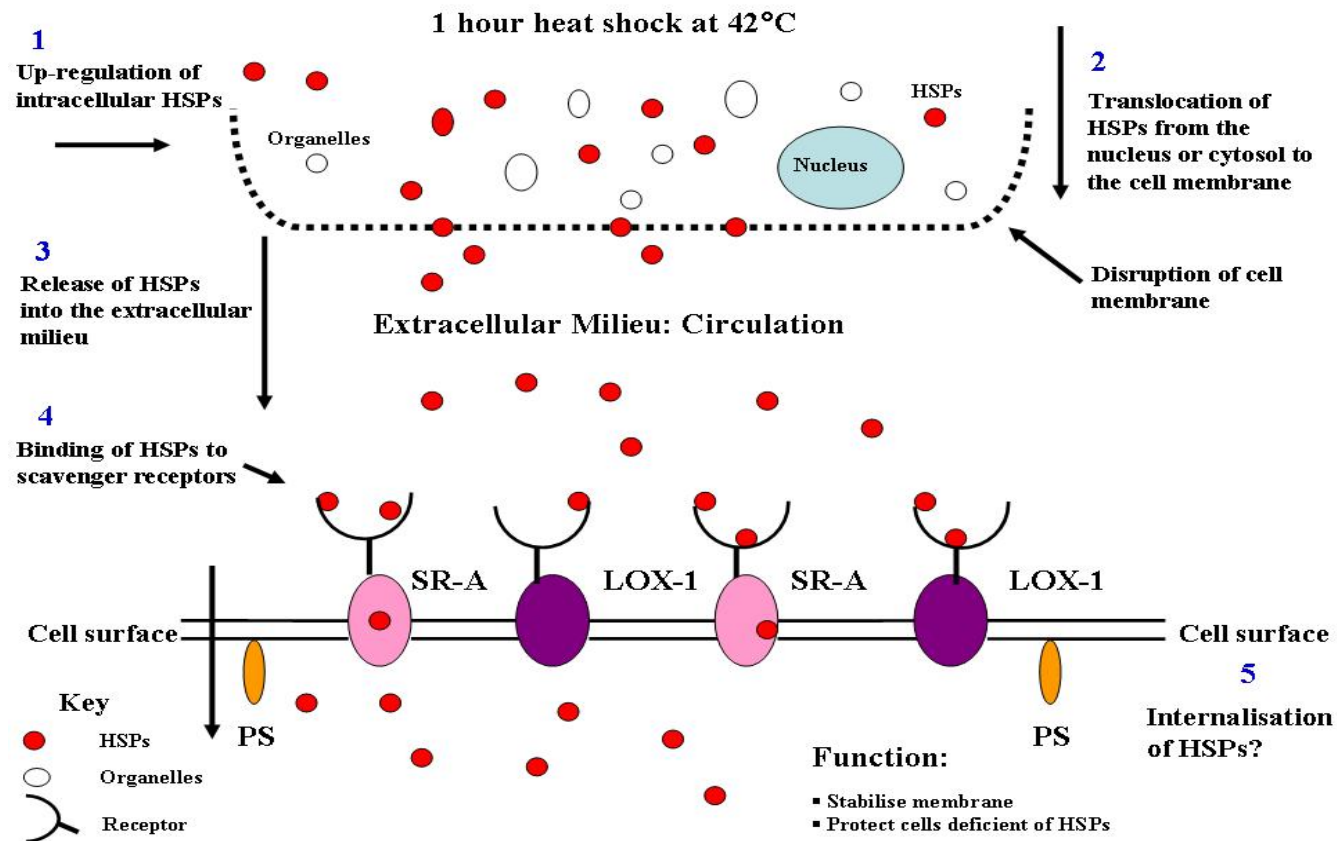
**Figure 7.1: The potential functions and consequences of cell surface expressed HSPs.**

Intracellular HSP translocation to the cell surface in response to elevated temperature stress, attraction of neighboring cells and/or immune cells *via* cell-to-cell signalling resulting in cellular protection and/or phagocytosis. (Adapted from Multhoff & Hightower, 1996; Ireland, 2009 and data demonstrated in this thesis).

### 7.3 Receptor interactions

The presence of internally derived HSPs on the cell surface of human leukocytes was affected following treatments with high salt concentrations, suggesting that the proteins are likely to be bound to a cell surface component. Previous evidence for the existence of HSP specific receptors on cell membranes has been shown by surface binding and uptake studies (Johnson et al. 1990, Guzhova et al. 1998; Arnold-Schild et al. 1999; Fujihara & Nadler, 1999; Guzhova et al. 2001; Yu et al. 2001; Novoselova et al. 2005), and in chapters four and five, in which the data supports the theory of HSP-mediated cell protection against factors inducing cell death (Guzhova et al. 1998; Guzhova et al. 2001). Previous studies suggest the protection results from the proteins being internalised (Houenou et al. 1996; Guzhova et al. 1998; Guzhova et al. 2001; Yu et al. 2001). However, the enhancement in protection from cellular stress in this thesis was not due simply to internalisation, but was more pronounced upon binding to the cell surface. Thus, the efficient binding of extracellular HSPs by the cells used in this study led to the assumption of a specific receptor on the cell surface. The fourth objective of this thesis therefore was to determine the surface receptors for extracellular Hsp72 on U937 and peripheral blood cells. The search for the extracellular HSP receptor is a topic of intense interest, and investigators have found evidence for Hsp72 receptors on a variety of innate immune cells including, macrophages (Asea et al. 2000b; Asea 2002), B cells (Arnold-Schild et al. 1999) and NK cells (Multhoff et al. 2001; Gross et al. 2003a). It can be speculated that HSPs bind to several receptors or even the plasma membrane itself. In this thesis, *in vitro* binding studies suggested an important role of members of the SR family in the interaction of cells with Hsp72. A comparative analysis found a significant expression of SR-A1 on U937 cells, whilst this receptor and LOX-1 were found on human leukocytes. Interestingly, these receptors were present on the cell surface under normal conditions and were highly up-regulated in response to conditions of elevated temperature stress. The selective binding of Hsp72 to SRs on human leukocytes led to the hypothesis that SRs might act as the interaction partner for Hsp72 on the plasma membrane of human erythrocytes. In line with these results, SR-A1 was identified on isolated erythrocytes. Thus, the results presented in this thesis (chapters four and five) suggest that the protection of cells from conditions of stress is highly regulated

by extracellular HSPs binding to SRs on the cell surface. This assumption was supported by *in vitro* competition experiments in which cells were pre-incubated with SR ligands prior to administration of extracellular HSPs, which were found to block the protective effects of these proteins. However, the possibility that Hsp72 may interact directly with membranes (Alder et al. 1990; Arispe et al. 2002; Negulyaev et al. 1996; Vigh et al. 1998) and bind several lipid components, in particular PS under conditions of stress as previously described (Hightower & Guidon, 1989; Arispe et al. 2002; Arispe et al. 2004), should not be excluded. Under normal conditions, PS resides on the cytosolic side of the plasma membrane, yet in response to stress or pre-apoptotic stages, PS translocates to the outer leaflet of the plasma membrane being exposed to the extracellular environment. The results of this study propose (chapter four) either, extracellular Hsp70 to interact with PS, Hsp70 to incorporate with PS and translocate to the cytosolic side of the plasma membrane or even a potential release of PS into the extracellular milieu, all revealed by a decrease in the binding of Annexin V to PS, but only during conditions of stress. These observations provided further evidence of a specific interaction of HSPs with PS lipids. It may be proposed *in vivo*, in response to an elevation of temperature PS may translocate, bind to the extracellular HSPs and then re-internalise by a rapid ATP-dependent mechanism transporting the HSPs at the same time. However, if PS serves as the natural binding partner for extracellular HSPs *in vivo* it would assume a high PS content on the outer membrane leaflet of normal cells. Since this study did not detect PS under normal conditions, it appears highly unlikely that PS serves as the dominant interacting partner for HSPs. The effect of heat shock treatment on the up-regulation, movement and release of intracellular HSPs into the extracellular milieu and the potential binding and/or internalisation of extracellular HSPs is summarised in figure 7.2. The SRs investigated in this part of study not only facilitate the binding and internalisation of extracellular HSPs during the stress response inducing cyto-protection, they are also up-regulated during macrophage differentiation due to extracellular HSP stimulation.



**Figure 7.2: The movement of intracellular HSPs in response to one hour treatment of elevated temperature stress at 42°C.**

HSP translocation from inside the cell to the cell membrane, its release into the extracellular milieu and potential binding to adjacent cells *via* SRs and/or internalisation resulting in membrane stabilisation and cellular protection. (Adapted from Calderwood et al. 2007b; Pockley, 2007 and data demonstrated in this thesis).

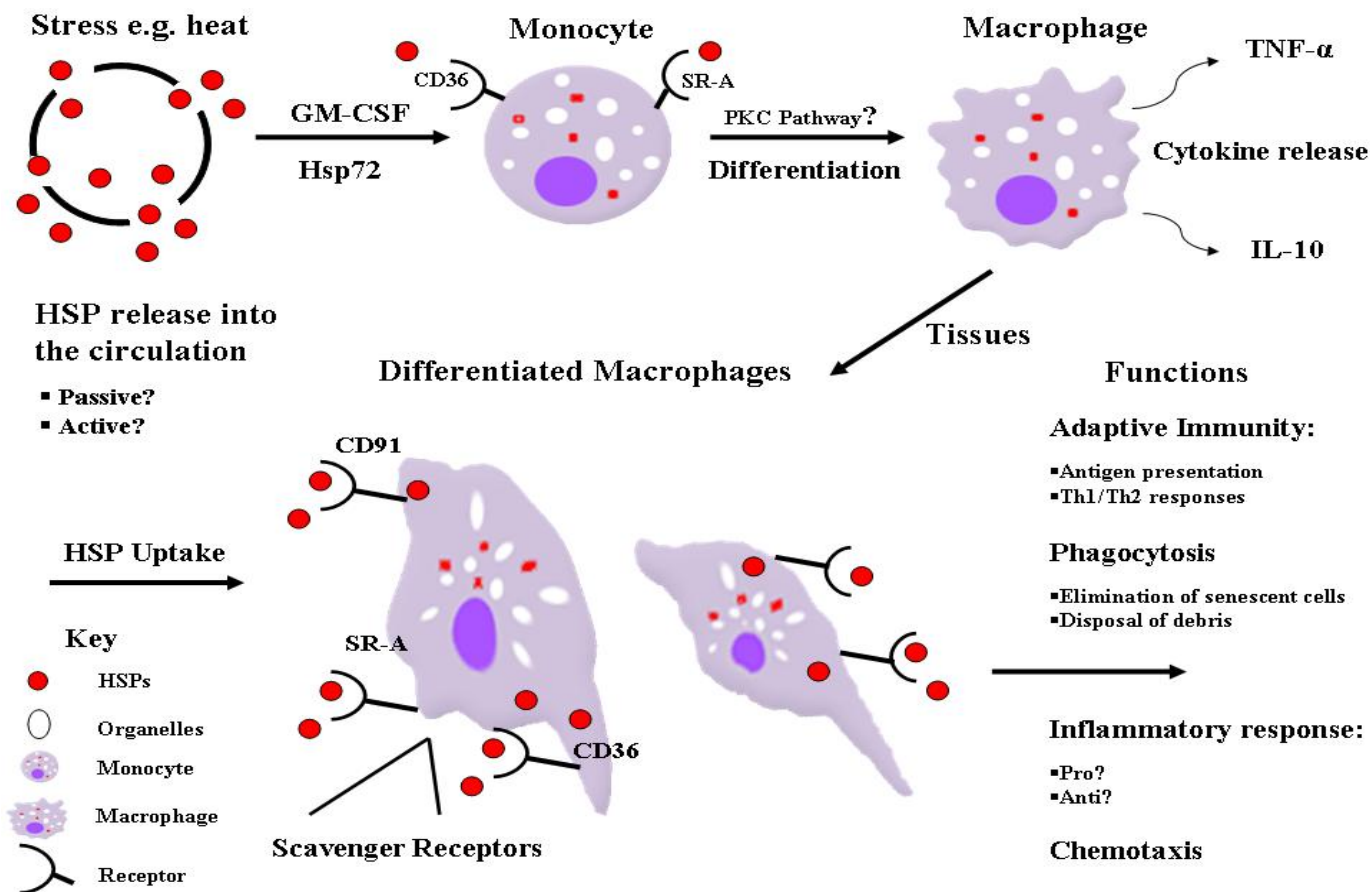
## 7.4 Immune regulation

Studies have shown that HSPs, when found in the extracellular environment act as immune regulatory signalling molecules which exert profound effects on the immune response. These include activation of target cells, particularly APCs such as DCs, APC maturation, cytokine production and stimulation of cytotoxic T cell responses (Gallucci et al. 1999; Kol et al. 2000; Sauter et al. 2000; Srivastava, 2002b), contributing to stress-induced innate immunity leading to acquired immunity. These effects are thought to be due to an interaction of extracellular HSPs with certain receptors on the cell surface, particularly SRs. Macrophages constitutively express a great variety of SRs, which allows them to respond to almost every single molecule produced during the stress response (Vega & De Maio, 2007). Thus, the final objective of this thesis was to determine whether extracellularly administered HSPs could stimulate an immune response. Extracellular HSPs (chapter six) were found to cause changes in the receptor pattern on the surface of U937 cells, which reflected the fact that these proteins could up-regulate the differentiation of U937 monocytes into U937 macrophages, as demonstrated through examining the surface expression of the differentiation markers: CD36, SR-A1, CD91 and CD14. Hsp72 in several forms: extracellular (bovine Hsp70 or human Hsp72), heat shocked U937 monocytes with increased levels of intracellular Hsp72 or Hsp72 enriched supernatant following a heat shock treatment (42°C or 46°C) of U937 monocytes, up-regulated all these surface markers with the exception of CD14, in a time- and dose-dependent manner, compared to treatment with/without the differentiation stimuli PMA. SR-A1 and CD91 were not as significantly induced compared to CD36 with extracellular HSPs and/or PMA, however all their values were collectively lower with extracellular HSPs in combination with PMA compared to treatment with PMA alone. The fact that the extracellular HSPs did not affect the expression of CD14 could reflect the possibility that certain selectively exists in the protein targets of HSPs. These results are in agreement with previous studies (Binder et al. 2000; Basu et al. 2001), who found incubation of macrophages with extracellular HSP (gp96) resulted in the up-regulation of several surface receptors, including those involved in phagocytosis, SR-A, CD14 and CD36. As suggested (chapter six), the most probable pathways by which PMA induces U937 differentiation is *via* the protein kinase-C pathway.

The addition of the extracellular HSPs prior to incubation with PMA significantly reduced the surface expression of these markers in a concentration-dependent manner when compared to cells stimulated with PMA alone. It may therefore be speculated that extracellularly administered HSPs, by masking surface structures, made PMA binding domains inaccessible with an inhibition of the whole protein kinase-C dependent mechanism (Pelech & Sanghera, 1992).

Human monocytes, isolated from healthy volunteers pre-incubated with extracellular HSPs and/or PMA *ex vivo*, also demonstrated an immune response, as evidenced by increased surface levels of the differentiation marker CD36. Thus, the observations in the cultured cell line are also operative in primary human monocytes, suggesting relevance *in vivo*. HSPs released from viable, stressed or damaged cells may activate human monocytes and stimulate their differentiation into macrophages. The up-regulation of the differentiation markers CD36, SR-A1 and CD91 may also have additional relevance aside from macrophage differentiation. Extracellular HSPs are proposed to act as 'danger signals' to the immune system when found in the extracellular milieu (Asea et al. 2000b; Matzinger, 2002; Campisi & Fleshner, 2003a; Campisi et al. 2003b; Svensson et al. 2006), thereby influencing a wide range of inflammatory reactions, hence they may activate cells and self-protecting mechanisms through stimulating the innate immune response and the inflammatory process. Through the induced expression of these markers which primarily function as SRs, they may recognise, initiate and facilitate receptor-mediated endocytosis of extracellular HSPs released from stress damaged or dead cells or even facilitate the uptake of cellular debris: bound cellular/chemical components, stimulating phagocytosis (Tani et al. 2009). This identifies a new role for Hsp72 in stimulating phagocytosis, thereby influencing both innate and adaptive immune responses. This suggests during monocyte differentiation the up-regulation of these differentiation markers may increase macrophage clearance of apoptotic and necrotic cells (Savill et al. 1992). When found in the extracellular milieu, HSPs always function as danger signals whether under normal or stressed conditions, however depending on their levels, the form of stress or whether they are chaperoning bound peptide will determine the level of an immune response. Regardless of the form of Hsp72 used in this study, these markers: CD36, SR-A1 and CD91 were up-regulated on the cell surface and an immune response was

generated, evidenced through the secretion of pro- and anti-inflammatory cytokines and also through functioning as chemoattractants for both U937 macrophages and human leukocytes. Thus, extracellular HSPs are proposed as being highly immunogenic. Macrophages become rapidly activated by the presence of pathogens such as bacteria and fungi, tumour and apoptotic cells and necrotic debris, which is followed by the digestion of the respective particle through phagocytosis. This results in an increase in survival as well as a decrease of cytokine production by macrophages, but causes the production of anti-inflammatory mediators. This study found increased levels of TNF- $\alpha$  in the supernatant of cells pre-incubated with extracellular HSPs up to twenty-four hours and increased levels of IL-10 after forty-eight hours, suggesting the secretion of the anti-inflammatory cytokine IL-10 suppressed the secretion of the pro-inflammatory cytokine TNF- $\alpha$  *in vitro*. This study is in agreement with other *in vitro* studies (Asea et al. 2000b; Wendling et al. 2000; Wang et al. 2001; Kakimura et al. 2002; Wang et al. 2005; Svensson et al. 2006; Wang et al. 2006). These *in vitro* studies are also supported by several *in vivo* studies suggesting a relationship between HSP and cytokines in disease, trauma or exercise (Kimura et al. 2004; Dybdahl et al. 2002; Bacelar, 2005). Furthermore, this function of extracellular Hsp72 is opposite to the function of intracellular Hsp72 which is able to inhibit the expression of pro-inflammatory cytokines and promote expression of anti-inflammatory genes IL-10 (Xian et al. 1999; Xie et al. 2002; Wang et al. 2002). It was also shown that extracellular HSPs stimulated the migration of both U937 macrophages and human leukocytes, speculating that extracellular HSPs may indeed function in inflammation promoting the release of regulatory proteins such as complement regulatory proteins: CR1, Crry or CD59 (Ródenas, Mitjavila & Carbonell, 1995). These results therefore provide further evidence that HSPs alone can act as highly stimulating agents for APCs, especially macrophages, resulting in the stimulation of a strong immune and hence inflammatory response, which may be of potential value in treating individuals with problems that affect the tissues including sepsis and burns (Kovalchin et al. 2006). The immune response to HSPs are summarised in figure 7.3.



**Figure 7.3: The transformation of human monocytes into macrophages *in vivo*.**

The differentiation of monocytes into the macrophage lineage orchestrated by extracellular Hsp72 binding to SRs on the cell surface and the effects of the immune response. (Adapted from Lindstrom, Miyazaki & Chang, 1997; Ireland, 2009 and data demonstrated in this thesis).



#### 7.4 Implications of the results and future work

This study provides evidence that the cell surface expression and extracellular location of certain HSPs can enhance the immune response through their inhibitory effects upon the production of pro-inflammatory cytokines, thus speculating immunogenic properties of peripheral blood cells. Both extracellular Hsp60 and Hsp70 in the peripheral circulation have been proposed to attenuate inflammatory disease such as RA, through inhibiting the production of the pro-inflammatory mediator TNF- $\alpha$  and NF- $\kappa$ B activation (Luo et al. 2008), or through stimulating immune regulatory T cell populations capable of down regulating inflammation (Wieten et al. 2007) or through producing regulatory TH2 cytokine responses (van Roon et al. 1997), thus suggesting anti-inflammatory and immune regulatory properties of extracellular HSPs as proposed in chapter six. Recent studies support the idea that administration of HSPs can be used directly as cytoprotective agents, and this study provides further evidence for this novel approach (chapters four and five), potentially of use in a wide variety of clinical situations such as transplantation and cell substitution therapy or involving traumatic injuries such as burns and possibly in chronic inflammatory or degenerative conditions such as RA, type-I diabetes and AD, where the viability of cells, tissues or organs must be supported. Several animal models have provided evidence of a possible therapeutic potential of extracellular HSPs. In adjuvant-induced arthritis in experimental rats a pre-immunization with HSP70 proteins protected animals from this experimentally induced disease, which resulted from the induction of IL-10 producing T cells that were capable of down-regulating inflammation (Wendling et al. 2000; Prakken et al. 2001; van Eden et al. 2005). Furthermore, in patients with type-I diabetes and polyneuropathy who have very low levels of Hsp72 in their blood leukocytes, Stokov and colleagues (2000) found administration of the anti-oxidant  $\alpha$ -lipoic acid which improves blood supply to the tissue, increased Hsp72 expression in the leukocytes of these patients and improved nerve damage (Stokov et al. 2000). It may be proposed therefore that administering extracellular HSPs through immunization or by enhancing release of intracellular HSPs into the extracellular milieu *in vivo*, perhaps *via* a sub-lethal stress, for example a heat shock treatment at 42°C as used in this study, that the binding and cellular uptake of these proteins (established in chapters four and five) points to membranes and

membrane-interacting compounds as future therapeutics. An advantage of the latter system is that the HSPs are human derived and therefore do not contain foreign material which may be of immunogenic potential. The uptake of extracellular HSPs and their delivery, perhaps in a HSP-peptide complex, to the cytoplasm and possibly the nucleus may be of important use as therapeutic agents since they remain localised in these intracellular sites for relatively long periods of time (chapter four).

Furthermore, extracellular HSPs may also be important in extending the lifespan of human erythrocytes *in vivo* in conditions of haemolytic anaemia, where it has been previously reported that high levels of Hsp72 are found internally and on the erythrocyte membrane (Zarić et al. 1998), in aplastic anaemia where patients have exaggerated heat shock response in terms of Hsp72 production (Takami et al. 1999), and in sickle cell disease, a condition in which there is an increase of serum Hsp72 following vaso-occlusive crisis (Adewoye et al. 2005). This association of elevated Hsp72 with anaemia and sickle cell disease suggests that the protein may have a role in maintaining membrane stability in these conditions and emphasises the importance of understanding the interaction with the erythrocyte membrane.

The work presented in this thesis focused on HSP induction and intracellular and/or extracellular localisation *in vitro* in a cultured cell line and *ex vivo* in a primary cell line in response to elevated temperature stress. It would be advantageous to examine the potential secretion and mechanism(s) of secretion of intracellular HSPs into the extracellular milieu *in vitro* and *ex vivo* following sub-lethal heat shock (42°C) induced translocation to the cell surface. In contrast, when examining the administration of extracellular HSPs *ex vivo* to human peripheral blood cells, further work is required to examine the localisation of HSPs with the plasma membrane and intracellular locations.

Despite the fact that this study has shown that extracellular HSPs prolonged the survival of human leukocytes from apoptotic/necrotic stress-induced cell death, further work must be done to determine the length of protection, also in terms of human erythrocyte survival it would be interesting to determine if the protection is localised or induced throughout the body. Further work in this area must also assess the contributions of other major HSPs: Hsp27, Hsp60 and Hsp90 for their

potential roles in cyto-protection and stimulating innate and adaptive immune responses.

In conclusion, this data presented in this thesis found that an elevation of temperature induces the simultaneous translocation of cell surface: membrane-bound or membrane-embedded expression of intracellular HSPs and PS, which rendered U937 cells and human leukocytes susceptible to apoptotic or necrotic cell death. In contrast, increasing whole cell HSPs provided these cells with cyto-protection. Furthermore, this thesis also established a role for extracellular HSPs in cyto-protection of U937 cells *in vitro* and peripheral blood cells *ex vivo*. The precise mechanism by which extracellular HSPs protect is not known, but this study suggests that it may be related to the capacity of the proteins to interact with the plasma membrane, by binding to a surface receptor and/or internalising. Regardless of the extracellular HSP used: bovine Hsp70 or recombinant human Hsp72 and the cells used: U937 cells or human leukocytes and erythrocytes, the protection was always associated with the binding to the cell surface, likely a receptor: SRs SR-A1 or LOX-1, or a lipid component such as PS on the cell membrane, however internalisation was not essential. These findings identify an essential role for extracellular HSPs and SRs in preventing heat shock induced apoptosis/necrosis and haemolysis.

This thesis also proposes functional consequences of extracellular HSPs in relation to the innate immune response, through stimulating monocyte to macrophage differentiation resulting in the up-regulation of CD36, SR-A1 and CD91 receptors, pro- and anti- inflammatory cytokine production and secretion (TNF- $\alpha$  and IL-10), and macrophage and human leukocyte migration. Hence, the results of this thesis strongly support a novel role for HSPs in cyto-protection and innate immune responses mediated through an interaction with the cell surface *via* SRs or PS. Thus, this thesis provides compelling evidence that extracellular Hsp72 is a potential target for therapeutic treatment of inflammatory and autoimmune diseases.

## Chapter 8

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